University of Alberta

The Role of RpoS in *A. vinelandii*: A Physiological and Proteomic Approach

By

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LIST OF ABBREVIATIONS AND SYMBOLS

ACP	Acyl Carrier Protein
BSA	Bovine Serum Albumin
CHAPS	3-[chloramidopropyl dimethylammonio]-1-propane-sulfonate hydrate
CFX	Cell Free Extract
Da	Daltons
DDT	dithiothreitol
dH_2O	distilled water
GC	Gas Chromatography
h	hour(s)
HPLC	High Performance Liquid Chromatography
IBD	Institute of Biomolecular Design
kDa	kiloDaltons
LC/MS/MS	Liquid chromatography mass spectrometry mass spectrometry
mA	milli-Amperes
Mb	mega base
Milli-Q-H_2O	distilled and deionized water
MS	Mass Spectrometry
MS/MS	Mass Spectrometry/Mass Spectrometry
NCBI	National Center for Biotechnology Information
PHA	Poly(3-hydroxyalkanoate)
PHB	Poly-β-hydroxybutyrate

PMSF	Phenylmethylsulfonyl fluoride
Q-ToF	Quadrapole-Time of Flight
rpm	revolutions per minute
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)aminomethane
UV	ultraviolet light
V	Volts
v/v	volume per volume
w/v	weight per volume

CHAPTER 1: INTRODUCTION

1.1 *Azotobacter vinelandii*-General Introduction

Azotobacter vinelandii is a large, aerobic, Gram-negative bacterium, which phylogenetically belongs to the gamma subgroup of proteobacteria. *A. vinelandii* is free-living and found in soil and water throughout the world (Su *et al.*, 1981). Recently the genome of this organism was sequenced and it was found to be 5.3 Mb in size with a GC content of 63.1% (www.azotobacter.org, 2003). The cell morphology of *A. vinelandii* is generally rod or ovoid in shape with cells arranged either singly or in pairs. The size of *A. vinelandii* is much larger compared to other bacteria having lengths of 1.5-2.0 μm or greater. *A. vinelandii* also possesses a number of interesting characteristics not commonly found in other bacteria. In addition to being naturally competent (Page and Sadoff, 1976) and a polyploid organism, *A. vinelandii* has the ability to form desiccation-resistant cysts, fix nitrogen non-symbiotically and aerobically and form complex polymers such as poly-β-hydroxybutyrate (PHB) and alginate and the unusual lipid alkylresorcinol, all of which will be discussed in this review.

1.2 Nitrogen Fixation

A. vinelandii is a non-symbiotic nitrogen-fixing bacterium that uses one of its three genetically distinct nitrogenases to fix nitrogen (Bishop, 1993, Bulen *et al.*, 1964). The energetically expensive nitrogen fixing reaction is given below:

$$8e^- + N_2 + 8\,H^+ + 18\text{-}24\ ATP \rightarrow 2NH_3 + H_2 + 18\text{-}24\ ADP + 18\text{-}24\ Pi$$

Bacteria from the genus *Azotobacter* were the first nitrogen-fixing organisms found to contain more than one type of nitrogenase, with the difference between the three types in *A. vinelandii* being the metal cofactors associated with the reactive centers of the protein complex (Kennedy and Toukdarian, 1987). The first enzyme complex is the well-characterized, conventional iron-molybdenum-containing nitrogenase that is only expressed when the organism is growing in a molybdenum-containing medium. The second nitrogenase is an iron-vanadium-containing enzyme complex that is formed when the organism is grown in medium lacking molybdenum, but containing vanadium. The third type of nitrogenase found in *A. vinelandii* does not contain either molybdenum or vanadium but uses iron as a metal cofactor and is made under molybdenum- and vanadium-deficient conditions (Bishop, 1993).

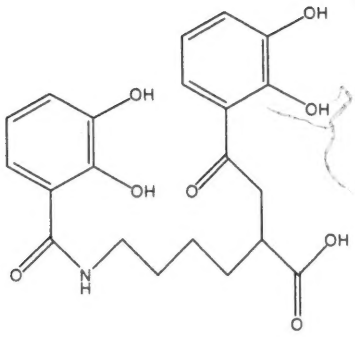
An important and unique feature of *Azotobacter* spp. fixing nitrogen is that they do so under aerobic conditions. Since the nitrogenase enzymes are irreversibly inactivated by oxygen, *Azotobacter* spp. have developed several mechanisms for protecting their nitrogenases against oxygen damage. These organisms have uniquely high rates of respiration coupled with a multitude of cytochromes and redox proteins. Multiple terminal oxidases consume oxygen in addition to coupling respiration to oxidative phosphorylation. Because of this "respiratory protection" the nitrogenase enzymes are maintained in an essentially anoxic environment inside the cell (Robson and Postgate, 1980). Recently it also has been speculated that the production of alginate, an industrially useful polymer produced by *A. vinelandii*, also plays a decisive role in protecting the nitrogenase enzymes. Alginate forms a capsule around *A. vinelandii* cells effectively forming a barrier for oxygen transfer into the cell (Sabra *et al.*, 2000).

1.3 Iron Acquisition

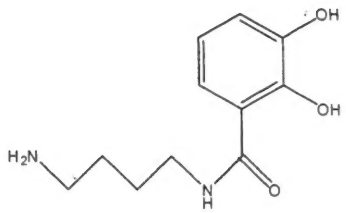
Iron is an essential nutrient for most organisms. *A. vinelandii* is capable of extracting iron from the environment by releasing low molecular weight iron chelators known as siderophores. Siderophores have a high affinity for Fe^{3+} and are essential because most organisms require iron at concentrations much higher than what is available in their immediate environment. Of the four siderophores *A. vinelandii* produces, three are catecholates; azotochelin (Corbin and Bulen, 1969), aminochelin (Page and von Tigerstrom, 1988), and protochelin (Cornish and Page, 1995) and one is the fluorescent pyoverdin-like siderophore, azotobactin (Demange *et al.*, 1986). The structures of these siderophores are shown in Figure 1.1. By making siderophores, *A. vinelandii* is able to solubilize iron and transport it into the cell (Page and Huyer, 1984). These iron-chelating siderophores are produced by *A. vinelandii* only under iron-limited conditions. Once in the cell, the iron plays a role in respiratory protection and as a cofactor in the enzymes superoxide dismutase, catalase, and nitrogenase (Tindale *et al.*, 2000)

1.4 Polyploidy

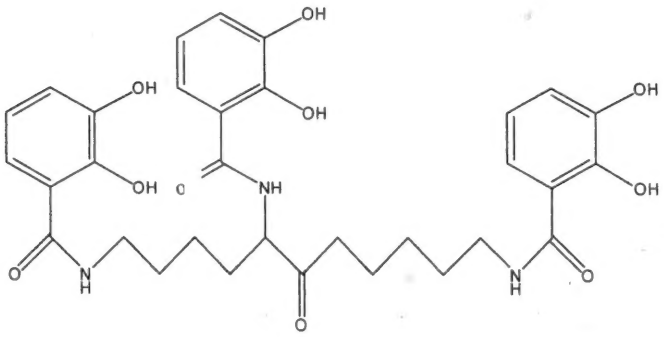
Physical studies of the *A. vinelandii* genome suggest this is a polyploid bacterium containing 40-80 chromosomes per cell (Sadoff *et al.*, 1979). This hypothesis has been used to explain the difficulty in isolating auxotrophic mutants in *A. vinelandii* while they are commonly found in other species. In a bacterium containing 40 or more chromosomes per cell, recessive mutants should be elusive because the long segregation



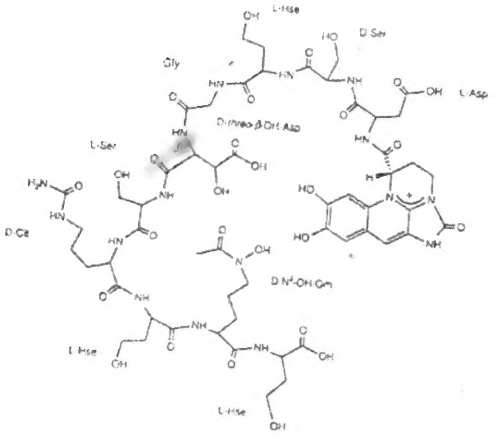
Azotochelin



Aminochelin



Protochelin



Azotobactin

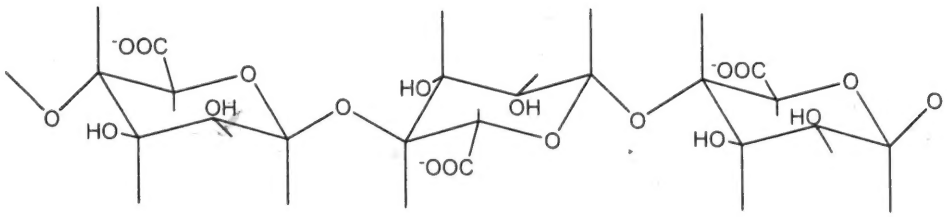
Figure 1.1 Siderophores produced by *A. vinelandii* (Cornish and Page, 1995; Page, 1993)

time required for their formation would cause loss of the mutation by dilution and recombinational repair (Maldonaldo *et al.*, 1992). There is, however, controversy in this area as some studies do not support polyploidy. One argument against polyploidy is the relatively easy isolation of mutants including *nif*- strains (Man-Hee Suh, 2001). This however is questionable because *nif* genes are non-essential as long as a nitrogen source is present, where as in essential genes the isolation of mutants is not possible without selection. Another argument is based on previous estimates of the size of the *A. vinelandii* genome which have been between 4.5 and 4.7 Mb. Recently however the *A. vinelandii* genome has been sequenced and it appears the size of the genome is actually around 5.3 Mb. Underestimating the size of the genome could possibly lead to an overestimate of the total number of chromosomes in an *A. vinelandii* cell. Studies by Maldonado *et al.* (1994) have suggested that polyploidy in *A. vinelandii* is growth phase and medium dependent. Their studies showed that during early exponential phase *A.vinelandii* had approximately 4 copies of its chromosome per cell whereas during late stationary phase up to 100 copies could be found per cell. Once encystment began, however, chromosome levels dropped back to the levels found in early exponential phase. *A.vinelandii* cells grown in soil extract medium did not show the same increase in the number of chromosomes per cell indicating polyploidy may actually be a laboratory artifact not found in nature (Maldonaldo *et al.*, 1994).

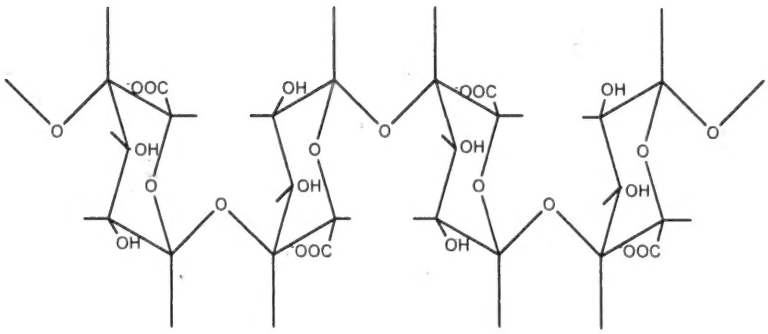
1.5 Secondary metabolites

1.5.1 Alginate

Alginate is a linear polysaccharide comprised of β-D-mannuronic acid and α-L-guluronic acid linked together by 1-4 linkages (Figure 1.2) (Rehm and Valla, 1997). The existence of alginate was first described by the British chemist E.C.C. Stanford who found that brown seaweeds produced abundant amounts of this polysaccharide. More than 80 years later microbial alginate was discovered by Linker and Jones (1964) who characterized it from a mucoid strain of *Pseudomonas aeruginosa* isolated from a cystic fibrosis patient. Two years later it was found that *A. vinelandii* also produces alginate (Gorin, 1966). Although *A. vinelandii* is capable of producing copious amounts of alginate, the alginate used in industry is generally obtained from harvesting brown seaweeds (Skjak-Braek *et al.*, 1986). The biochemistry and genetics of alginate biosynthesis in *A. vinelandii* are similar to the well-studied bacterium *P. aeruginosa*. These studies are motivated by the pathogenesis of the latter bacterium. *P. aeruginosa* mucoid strains, which produce great amounts of alginate, are the most important cause of death of cystic fibrosis patients (Rehm and Valla, 1997; Campos *et al.*, 1996). The production of alginate in this organism not only protects it from external conditions by forming a coat around the cell, but it also enhances its adhesion to solid surfaces. As a result of this adhesion, a biofilm develops which is advantageous for the survival of the bacterium in the lung (Sabra *et al.*, 2001). Industrially, interest in alginate lies in its potential application as a stabilizing, thickening and gelling agent in food production or to immobilize cells in pharmaceutical and biotechnology industries (Rehm and Valla, 1997). There are many other potentially useful applications for alginate, all of which are



Manuronic acid



Guluronic acid

Figure 1.2 Structure of alginate (Rehm and Valla, 1997)

summarized in Table 1.1. Due to the potential use of alginate as a food or pharmaceutical additive and due to the hazards of the pathogen *Pseudomonas* spp., *A. vinelandii* is a better choice for microbial alginate production. Also, alginate containing blocks of polyguluronic acid or G-blocks forms the most rigid and useful type of polymer. G-blocks actually form a structured "egg box conformation" (Figure 1.2) which is stabilized by calcium ions. *Pseudomonas* spp. do not have the ability to form G-blocks as they do not have the epimerases required to catalyse the conversion of manuronic acid to guluronic acid (May and Chakrabarty, 1994). *A. vinelandii* has six epimerases involved in this conversion (Hilde, 2000), and these may produce novel arrangements of G-blocks giving different alginate properties.

1.5.2 Alginate Biosynthesis

During the formation of alginate, fructose-6-phosphate is first converted to mannose-6-phosphate by the enzyme phosphomannose isomerase, the product of the *algA* gene (Figure 1.3). Mannose-6-phosphate is then converted to mannose-1-phosphate and subsequently GDP-mannose by the products of the *algC* (phosphomannomutase) and *algA* (GDP-mannose pyrophosphorylase) genes. The GDP-mannose is then catalysed to GDP-manuronic acid by GDP-mannose dehydrogenase encoded by *algD*. The final step in alginate production is the conversion of GDP-manuronic acid, which is the immediate precursor for polymerization, into alginate by AlgG epimerase. (Wang *et al.*, 1987). To excrete alginate out of the cell, the pore-forming protein encoded by *algE* is used.

Table 1.1 Applications of alginates produced commercially (Rhem and Valla, 1997; Onsoyen, 1996)

Application	Function of alginate
Textile printing	Fixation, color yield and brightness, ensuring even printing
Paper and board treatments	Improvement of surface uniformity
Welding-rod production	component of covering materials for welding rods, improving bendability
Water treatment	Increasing aggregate sizes in flocculation processes
Can sealing	Formation of cords into slits of can lids
Creaming of latex	Concentration of natural latex during extraction from rubber plants
Production of ceramics; food for humans and pets	Reduced rates of surface drying; stabilizing, thickening and gelling agent
Pharmaceutical and biotechnology industry	Immobilization of cells, sustained release, treatment of oesophageal reflux, dermatology and wound healing, dental-impression materials

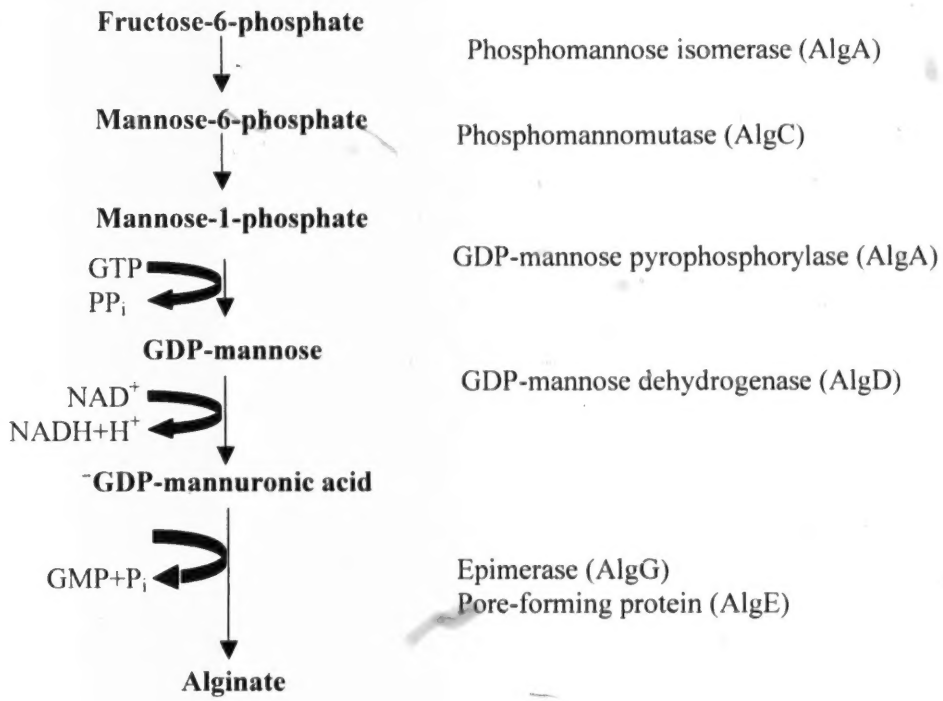


Figure 1.3 Biochemical pathway for alginate production (Sabra *et al.*, 2001)

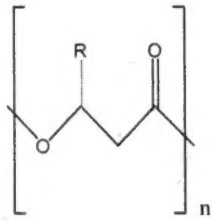
Extracellular polymannuronic acid C-5-epimerase then catalyses the conversion of mannuronic acid in the polymer to guluronic acid residues (Sabra *et al.*, 2001).

1.5.3 Poly(3-hydroxyalkanoates) Production in Bacteria

Poly-3-hydroxyalkanoates (PHAs) are produced as intracellular lipid storage material by many Gram positive and Gram negative bacterial species (Ren *et al.*, 2000). Bacteria produce these insoluble molecules especially when nutrient supplies are imbalanced. PHAs have been found to occupy as much as 90% of cell dry weight in some bacteria. The role of these compounds is to serve as a sink for carbon and as reducing equivalents (Madison and Huisman, 1999). By polymerizing soluble intermediates into insoluble molecules, like PHA, the cells do not alter their osmotic state and the leakage of these valuable energy sources is prevented (Madison and Huisman, 1999). These compounds have also attracted considerable attention due to their potential applications as biodegradable plastics. When extracted from the cell and processed, PHAs have properties that are similar to some common plastics such as polypropylene (Byrom, 1987). Since PHAs are excellent carbon and energy sources, many microorganisms have evolved the ability to break down these macromolecules in aerobic and anaerobic environments (Chen and Page, 1994). PHAs are also recyclable like the petrochemical thermoplastics (Madison and Huisman, 1999)

There are many different types of PHAs. The most common ones that have been identified are linear, head to tail polyesters composed of 3-hydroxy fatty acid monomers.

In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of a neighboring monomer:



The "R" group, or alkyl group can vary from methyl (C_1) to tridecyl (C_{13}). There have also been reports of aromatic, unsaturated, halogenated, epoxidized and branched monomers within this alkyl side group (Madison and Huisman, 1999). The composition of the PHA depends on the carbon source, the PHA synthases and the metabolic routes involved (Ren *et al.*, 2000). This variation in length and composition of the side chain accounts for the diversity of the PHA polymer family, which leads to an extensive array of chemical and physical properties and potential applications.

PHAs have been classified into two groups. The first group includes the short chain length PHAs, containing 3-hydroxylalkanoate monomers with chain lengths C_4-C_5. The second group, the medium chain length PHAs, are characterized by 3-hydroxyalkanoates with chain lengths C_6-C_{14} (Ren *et al.*, 2000). These compounds are synthesized from fatty acids or other aliphatic carbon sources. Most bacteria form short chain length PHAs. A notable exception is the pseudomonads, *P. aeruginosa* and *Pseudomonas putida,* which both form medium chain length PHAs. Fatty acid metabolism, both biosynthesis and β-oxidation routes, provide intermediates for the formation of medium chain length PHAs in these organisms as can be seen in Figure 1.4

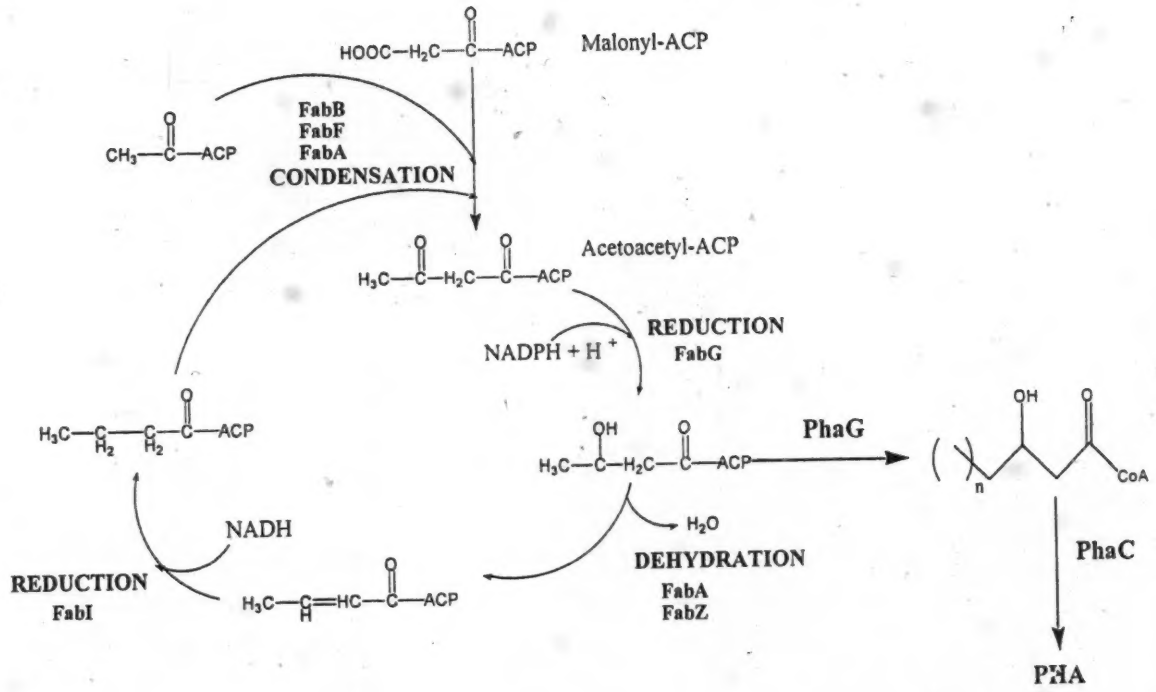


Figure 1.4 Biosynthetic pathway for medium-chain-length PHAs from lipids. PhaG is an acyl-ACP:CoA transacylase. (Madison and Huisman, 1999).

(Madison and Huisman, 1999; Page and Manchak, 1995). Non-growing cultures of *P. putida* are able to synthesize PHA from either glucose or fatty acids when these carbon sources are in excess. When a fatty acid synthesis inhibitor is added to these cultures however, no PHA is formed from glucose confirming the formation of PHA from glucose is linked to fatty acid biosynthesis. In the same fashion, if a β-oxidation blocker is added to cultures, the formation of PHA from octanoate is prevented, but not formation of PHA from glucose (Madison and Huisman, 1999).

PHAs are most commonly represented by poly-β-hydroxybutyrate also known as PHB. PHB was discovered by Lemoigne in 1926 at the Pasteur Institute, making it the first PHA to be discovered and likely contributing to its popularity among researchers. This polymer can be a useful taxonomic tool when identifying bacterial species (Madison and Huisman 1999; Page and Manchak, 1995).

Although PHB has potential applications as a bioplastic, the plastic it forms is too brittle to be of any great commercial value. To overcome this, valerate is added to the medium where it metabolized to β-hydroxyvalerate by β-oxidation in the bacterium and incorporated into PHA synthesis (Figure 1.5). The resulting copolymer is poly-β-hydroxybutyrate-co-β-hydroxyvalerate which is less stiff and brittle than PHB. This new molecule is a thermoplastic that can be processed at a lower temperature than PHB making it easier to work with. Poly-β-hydroxybutyrate-co-β-hydroxyvalerate still retains the novel mechanical properties of PHB and can be used to prepare films with excellent water and gas barrier properties, similar to those of polypropylene (Madison and Huisman, 1999).

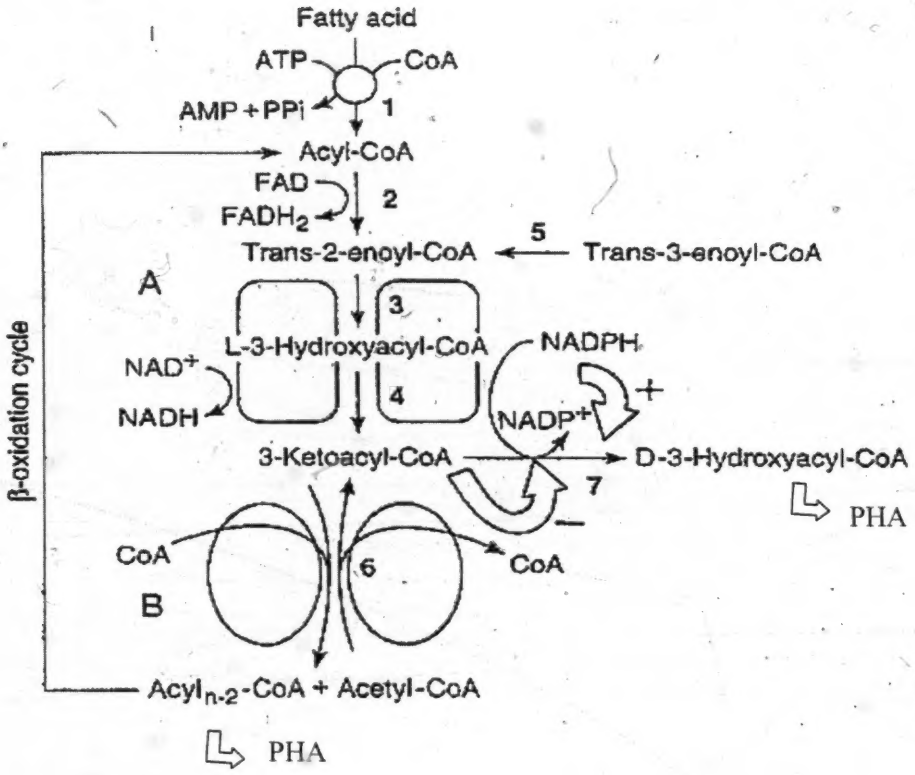


Figure 1.5 β-oxidation provides intermediates for bacteria to synthesize PHAs . (Page and Manchak, 1995).

An important characteristic of PHAs is their biodegradability. Many microorganisms secrete extracellular PHA hydrolases and depolymerases, thus having the ability to break down PHAs. The activity of these enzymes varies depending on the composition of the polymer, the amount of polymer present and the environmental conditions. The degradation of poly-β-hydroxybutyrate ranges from approximately a few months in anaerobic sewage sludge to a few years in sea water (Madison and Huisman, 1999). PHA plastics are also part of the normal carbon cycle and therefore a renewable resource (Page and Manchak, 1995). The fermentative production of PHAs uses agricultural products such as sugars and fatty acids as carbon and energy sources. These carbon sources are derived from CO_2 and water, and after their conversion to biodegradable PHA, the breakdown products again are CO_2 and water.

1.5.4 PHB in *A. vinelandii*

A. vinelandii produces the short chain length intracellular lipid polymer PHB. In *A. vinelandii* this polymer has a high molecular weight of about $1x10^6$ Da (Chen and Page, 1994). In addition to the production of this high molecular weight intracellular storage polymer, Reusch *et al.* (1987) have shown that *A. vinelandii* forms a low molecular weight PHB polymer. The incorporation of this low molecular weight PHB into the *A. vinelandii* cell membrane coincides with the development of a sharp

lipid phase-transition. It has been suggested that this low molecular weight PHB complex alters the barrier properties of the membrane and is responsible for the formation of non-bilayer lipid structures seen at the onset of genetic competence and encystment (Reusch et al., 1987).

1.5.5 PHB Biosynthesis

Three enzymes are involved in PHB biosynthesis in *A. vinelandii*: β-ketothiolase, acetoacetyl-CoA reductase and PHB synthetase. These enzymes are encoded by the genes *phbA, phbB* and *phbC,* respectively. All three genes are part of the *phbCAB* operon (Fig 1.6) (Madison and Huisman 1999). PHB is synthesized in a three-step pathway by the successive action of these enzymes (Figure 1.6) (Madison and Huisman 1999). PHB synthesis in *A. vinelandii* is regulated at the level of the β-ketothiolase activity, the first step in the biosynthesis pathway (Castaneda *et al.*, 2000; Manchak and Page, 1995). Under aerobic conditions, acetyl-CoA is fed into the tricarboxylic acid cycle, releasing CoA which in turn inhibits β-ketothiolase activity and ultimately PHB synthesis. When oxygen is limited however, NADPH increases and inhibits citrate synthase and isocitrate dehydrogenase. This lowers the levels of free CoA, allowing the synthesis of PHB to proceed (Page and Manchak, 1994; Segura and Espin, 1998). Figure 1.7 further illustrates this control of PHB synthesis in *A. vinelandii*.

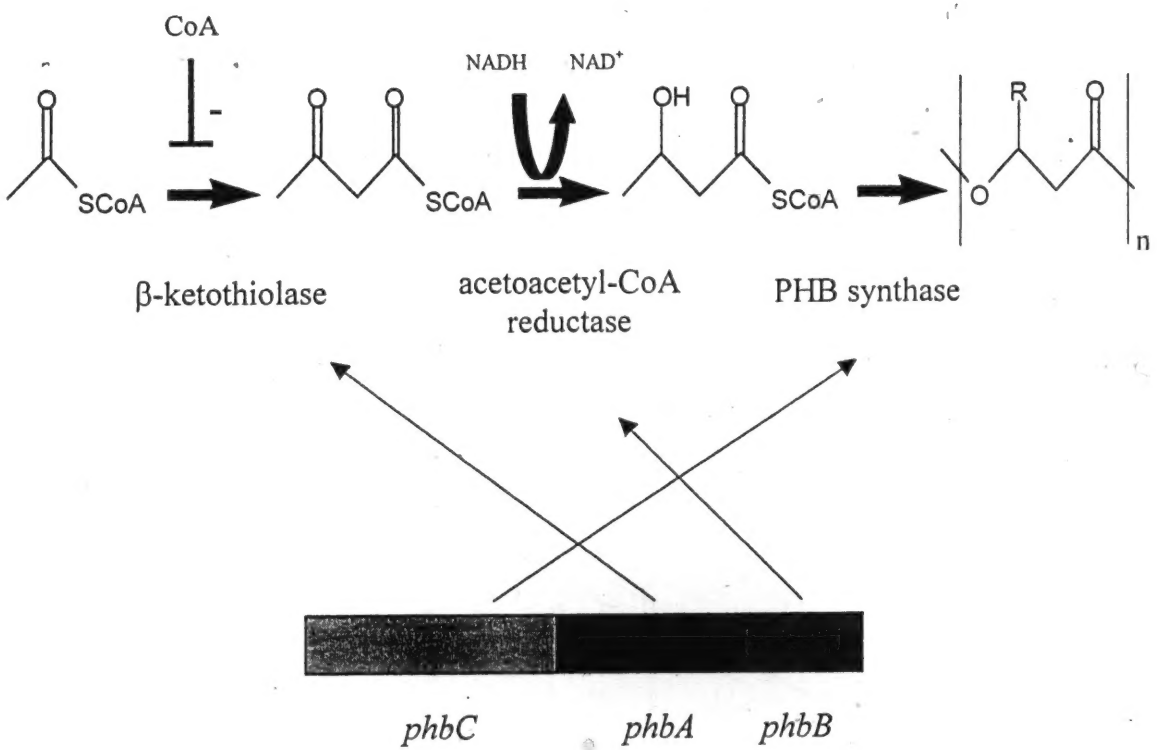


Figure 1.6 Biosynthetic pathway for PHB and organization of the *phaCAB* operon in *Zoogloea ramigera* (Madison and Huisman, 1999).

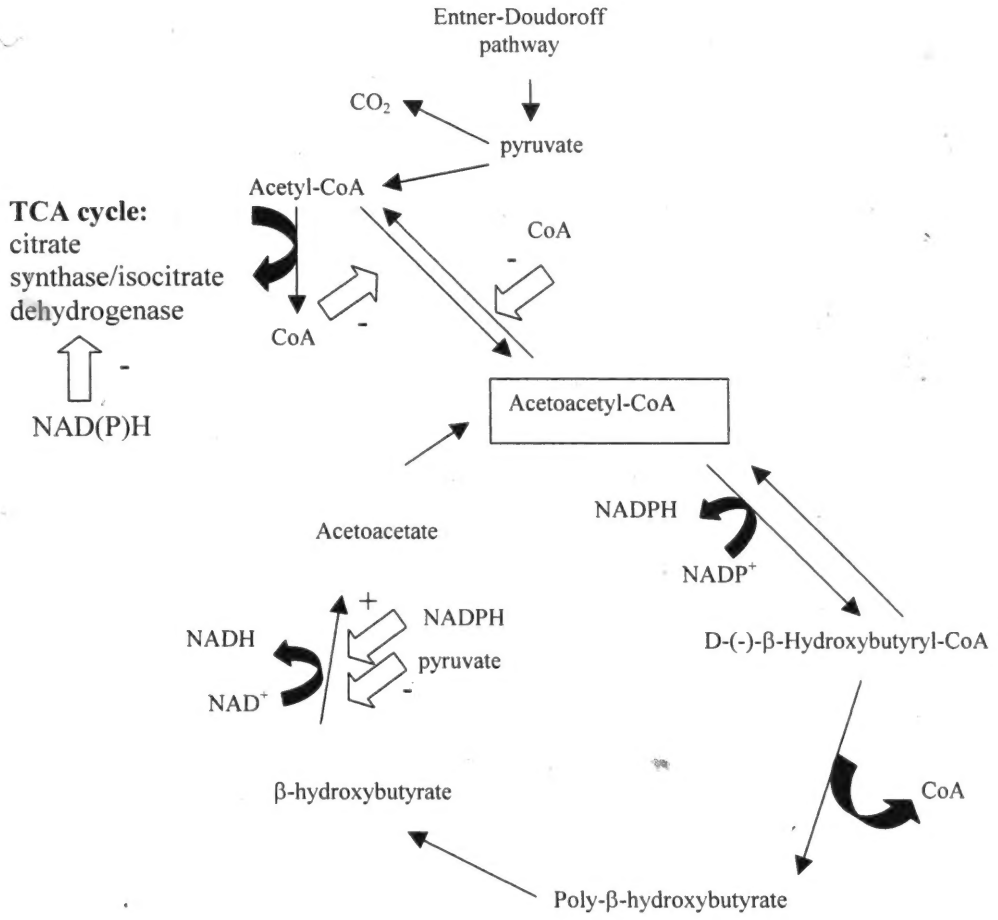


Figure 1.7 Control of PHB synthesis in *A. vinelandii* Block arrows indicate areas where positive (+) or negative (-) control exists. (Page and Manchak, 1995).

1.5.6 Alkylresorcinols

Resorcinolic lipids are natural, amphiphilic, long-chain, odd-numbered homologs of orcinol (1,3-dihydroxy-5-methylbenzene) that exhibit a high affinity for lipid bilayer and biological membranes (Kozubek *et al.*, 1996). Numerous plant species and microbial organisms, including *A. vinelandii,* produce these phenolic compounds. The general structure of alkylresorcinols is shown in Figure 1.8. Alkyresorcinols with C_{21} side chains were first found in *A. vinelandii* by Reusch and Sadoff in the lipids of encysting cells (Reusch and Sadoff, 1983; Reusch and Sadoff, 1979) (See Section 1.6). Further experiments showed that during encystment of *A. vinelandii*, over 70% of lipids were replaced with resorcinolic and other phenolic lipids with alkylresorcinols making up half of these phenolic compounds (Su *et al.*, 1981). The biological role of these compounds in bacterial organisms is not yet fully understood. However, it is suggested they are autoregulatory anabiotic compounds due to their increased levels during encystment and alteration of cellular metabolism, more specifically lipid metabolism (Nenashev *et al.*, 1994; Reusch and Sadoff 1979). Bitkov *et al.* (1992), investigated the mechanism by which alkylresorcinols affect the lipid bilayer of cellular membranes. To do this, they formed planar bimolecular membranes from alkylresorcinols and from mixtures of typical bacterial phospholipids. They found the alkylresorcinols associated with the phospholipids forming oligomeric and polymeric complexes, giving rise to modifications in the bilayer structure and properties. Alkylresorcinols at micromolar concentrations also have been shown to be active antioxidants, which protect both free fatty acids and phospholipids against ferrous ion-induced peroxidation (Gasiorowski *et al.*, 1996).

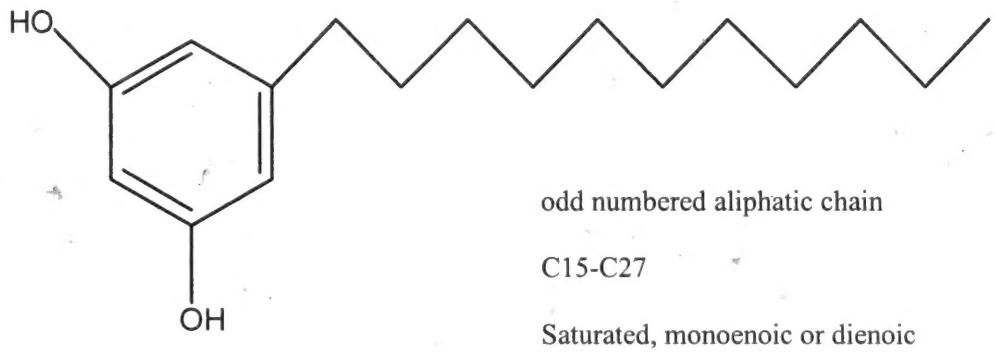


Figure 1.8. General Structure of Alkylresorcinol (Gasiorowski *et al.*, 1996)

1.6 Encystment

Cysts are produced by *A. vinelandii* after the termination of exponential growth when environmental conditions are unfavorable. By forming cysts, *A. vinelandii* is able to survive long periods of extreme nutrient limitation and desiccation (Socolofsky and Wyss, 1962; Moreno *et a.l*, 1986). Cysts display a significant rate of exogenous respiration, however far from that of the vegetative cell (Socolofsky and Wyss, 1961; Parker and Socolofsky, 1966). Cysts also contain high levels of calcium relative to vegetative cells and have been found to sustain viability after 24 years of storage in dry soils (Thompson and Skerman, 1979), although they do not exhibit the heat resistance observed in bacterial endospores (Moreno *et al.*, 1998; Sadoff, 1975). Vegetative cells of this species are motile by means of peritrichous flagella, however, upon induction of encystment, the cells lose motility (Figure 1.9). During encystment, cells change from carbohydrate metabolism to lipid metabolism and their cell walls thicken, becoming quite spherical (Sadoff, 1975; Su *et al.*, 1981). Cysts typically contain half the volume of a vegetative cell but twice the amount of lipid on a dry weight basis. A wide variety of fatty acids are found in cysts whereas only a few fatty acids are found in vegetative cells (Su *et al.*, 1981). Alginate, PHB and alkylresorcinols are all involved in the encystment process. The cysts of *A. vinelandii* are surrounded by two capsule-like layers, known as the intine and the exine, which both contain a high proportion of alginate (Lloret *et al.*, 1996). This polysaccharide coating is what protects the cysts from desiccation. The intine or inner layer is made up of mostly polymannuronic acid residues whereas the exine or outer layer is made up of mostly polyguluronic acid resulting in greater stiffness (Sadoff, 1975). Under favorable conditions the alginate coating swells and the cyst

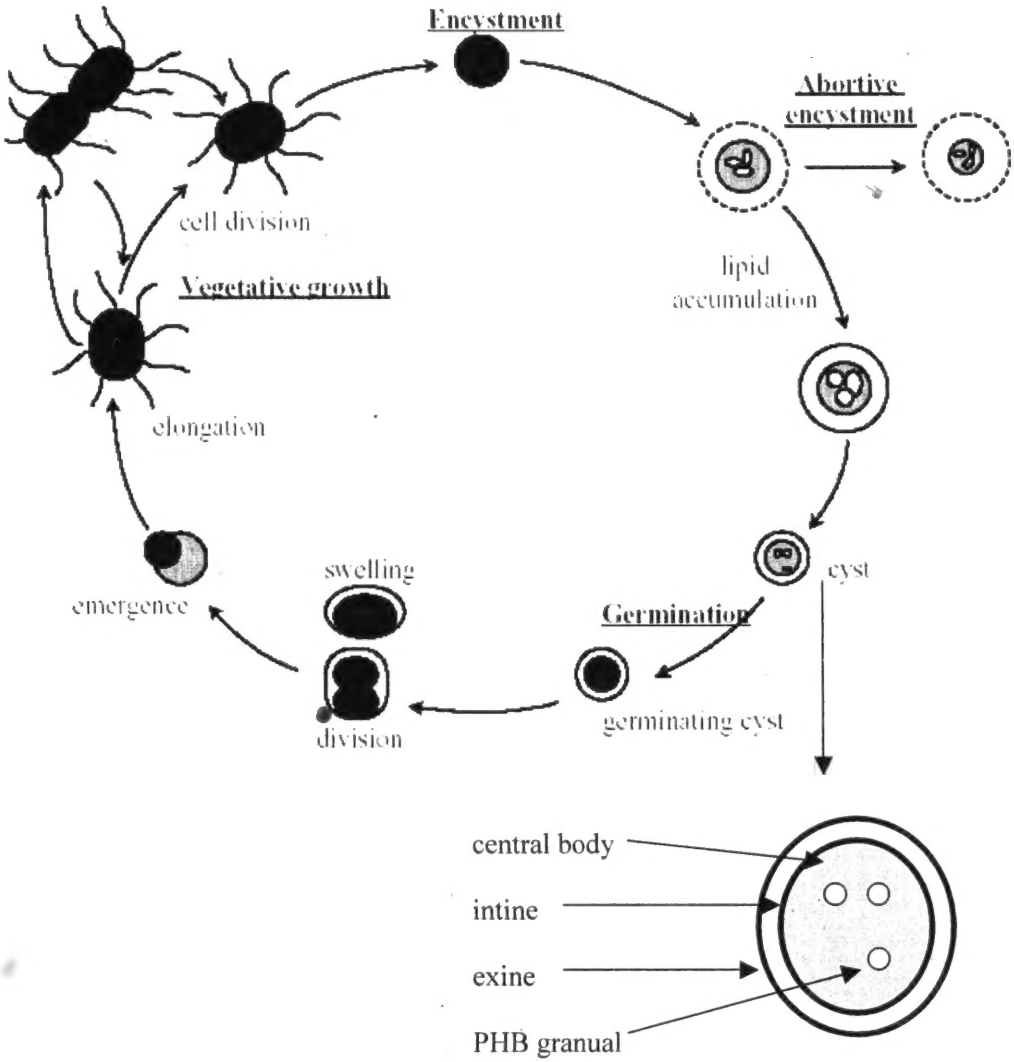


Figure 1.9. Life cycle of *A. vinelandii* showing cyst structure. (Sadoff, 1975)

germinates, thus re-entering the vegetative state. Low molecular weight PHB has also been shown to be incorporated into the cell membrane, coinciding with the development of cysts (Reusch *et al.*, 1987) (Also see Section 1.5.2). In addition, alkylresorcinols have also been shown to associate with the lipid bilayer during encystment (See Section 1.5.6).

1.7 Stress Response in Bacteria

In order for microorganisms to survive their environment they must be able to respond appropriately to stress. In any environment a microbe may be exposed to extreme temperatures, varying pH, toxic compounds or lack of nutrients, all of which require some sort of adaptation by the organism.

As a soil bacterium, *A. vinelandii* has to be able to respond to external stresses. Soil environments are characterized by the presence of many organic substrates, chemical gradients and varying concentrations among nutrients. Also, since one gram of soil contains up to 10^{10} bacterial cells and 10^{4} bacterial species, complex microbial interactions also must be taken into account (Torsvik, 1996). One method of dealing with stress used by *Escherichia coli* as well as *A. vinelandii* and most other bacteria is the alteration of the core RNA polymerase. This is done in order to allow the RNA polymerase to recognize specific promoter regions and transcribe genes when their products are required for survival during times of stress. Alterations in the core RNA polymerase occur through the binding of sigma factors.

1.8 Sigma Factors

Sigma factors are proteins that bind the core RNA polymerase and introduce a major change in the affinity of the RNA polymerase for DNA. Only the holoenzyme complex is able to initiate transcription and the sigma factor ensures the bacterial RNA polymerase binds in a stable manner to DNA only at promoters. The sigma factor is released when the RNA chain reaches eight or nine bases leaving the core enzyme to undertake elongation (Lewin, 1994). Sigma factors, in *E. coli*, generally allow the RNA polymerase to recognize a hexameric sequence 10 bases upstream of the transcription start site, as well as a hexameric sequence 35 bases upstream from the transcription start site. These sites are known as the –10 and –35 regions respectively (Lewin, 1994).

The *E. coli* genome encodes at least six different sigma factors, each of which directs the RNA polymerase to a different set of promoter sequences (Neidhardt, 1996). The best-studied sigma factor, σ^{70} or RpoD, is required for the transcription of genes involved in normal or "house keeping" functions like metabolism and biosynthesis during growth in exponential phase. Other sigma factors are responsible for coordinating transcription of functionally related sets of genes including those involved in heat shock response (σ^{32}, or RpoH and σ^{24} or RpoE), nitrogen assimilation (σ^{54} or RpoN), flagellum gene expression (σ^{28} or RpoF) and stationary phase or stress-induced gene expression (σ^{S} or RpoS) (Neidhardt, 1996). These different types of sigma factors can be placed into four categories: primary sigma factors which include RpoD and RpoF; alternative sigma factors like RpoE and RpoS involved in heat shock or other stress responses; Extracytoplasmic Function (ECF) sigma factors which are responsible for directing

extracytoplasmic functions in response to extracellular stimuli (Lonetto et al, 1994) and finally RpoN which shows low homology to the other sigma factors (Neidhardt, 1996).

1.9 RpoS

RpoS is an alternative sigma factor found to date only in the γ subgroup of proteobacteria. RpoS was discovered independently by several groups and is induced during stationary phase as well as during other times when cells are subjected to stress. In *A. vinelandii*, RpoS is 334 amino acids in length, sharing 76% identity with RpoS from *E. coli* and 87% identity with *P. aeruginosa* and *P. putida* (S. Kujat-Choy, unpublished). Currently, RpoS is known as the central regulator of bacterial stress responses and has even been considered "the master regulator of the general stress response in *E. coli*" (Becker *et al.*, 1999). RpoS is known to exert control over at least 70 different genes in *E. coli*, including genes responsible for conferring resistance against oxidative stress, near-UV irradiation, heat shock, hyperosmolarity, acidic pH and ethanol. RpoS also controls gene products that change overall cell morphology; for example, stressed *E. coli* cells tend to become smaller and ovoid. Metabolism is also affected by RpoS in the switch from a maximal growth metabolism to a maintenance metabolism (Hengge-Aronis, 2002). Interestingly, RpoS also is involved in controlling the genes that mediate programmed cell death in stationary phase. This increases the chances for survival of an entire bacterial population under extreme stress by sacrificing a portion of the population in order to provide nutrients for the survivors (Bishop *et al.*, 1998). In enteric and pathogenic bacteria it has been found that many virulence genes are under the control of

RpoS. Naturally this is to be expected as host organisms generally provide stressful environments to evade pathogens.

Much remains to be learned about the physiology of the RpoS response. The same can be said for the regulatory network directed by RpoS. To date, however, the basic regulation of RpoS is reasonably well understood with multiple levels of control affecting transcription, translation, and post-translational regulation.

1.10 Regulation of RpoS

Regulation of RpoS has been considered one of the most complex system in bacteria, involving transcription, mRNA turnover, translation initiation and proteolysis (Figure 1.10). At the level of transcription, *rpoS* is stimulated by controlled downshifts in growth rate in a chemostat (Notley and Ferenci, 1996). When growth is abruptly stopped however, there is only a weak increase in *rpoS* transcription (Lange and Hengge-Aronis, 1991; Lange and Hengge-Aronis, 1994). The rate of translation of already existing *rpoS* mRNA is increased by high osmolarity, growth at moderately low temperatures, as well as when cultures reach a certain cell density during growth in minimal glucose medium and also in response to a pH downshift (Lange and Hengge-Aronis, 1994). The RpoS protein is also controlled at the level of degradation. In cells growing on minimal medium, RpoS is degraded with a half-life between one and three minutes. When cells are responding to a stress like starvation or hyperosmolarity, RpoS proteolysis is considerably reduced or even stopped completely and as a result, RpoS accumulates in the cell rapidly (Lange and Hengge-Aronis, 1994).

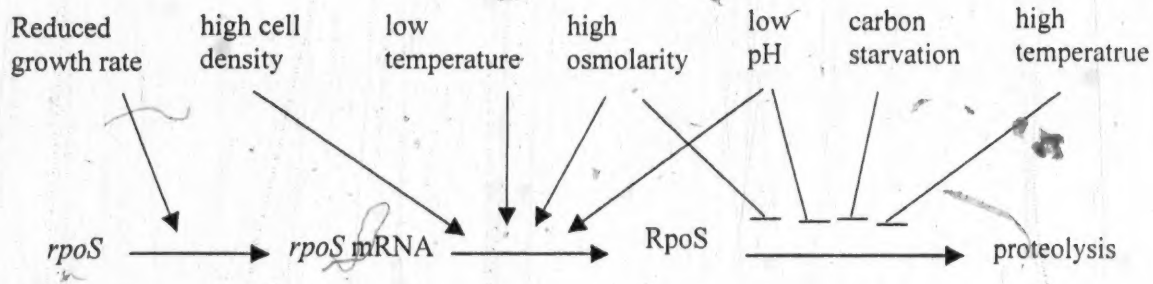


Figure 1.10. Various levels of RpoS regulation. (Hengge-Aronis, 2002)

1.11 Proteomics

The term proteomics was coined in 1995 (Anderson and Anderson, 1996), however the first studies that can be called proteomics began 20 years earlier with the introduction of the two-dimensional gel (O'Farrell, 1975). Currently there are many different areas of study now grouped under the term proteomics (Figure 1.11) including protein-protein interaction, protein modifications, protein function and protein localization to name a few. The aim of proteomics is not only to identify all the proteins in a cell, but also to create a three-dimensional map of the cell indicating where these proteins are located (Graves and Haystead, 2002).

There are limitations to studying only genomic information. Genomic information is static whereas the information provided by proteins is dynamic. While the studying of DNA and RNA is useful, they cannot tell you if or when or under what conditions a protein will be in its active form. Multiple proteins can be obtained from each gene when post-translational modifications are taken into account. Also, DNA and RNA analysis cannot predict the amount of a gene product that is made, or if and when a gene will be translated. The study of the proteome of a cell reflects the immediate environment in which it is studied. In response to internal or external cues, proteins can be synthesized, modified or translocated within the cell or be degraded.

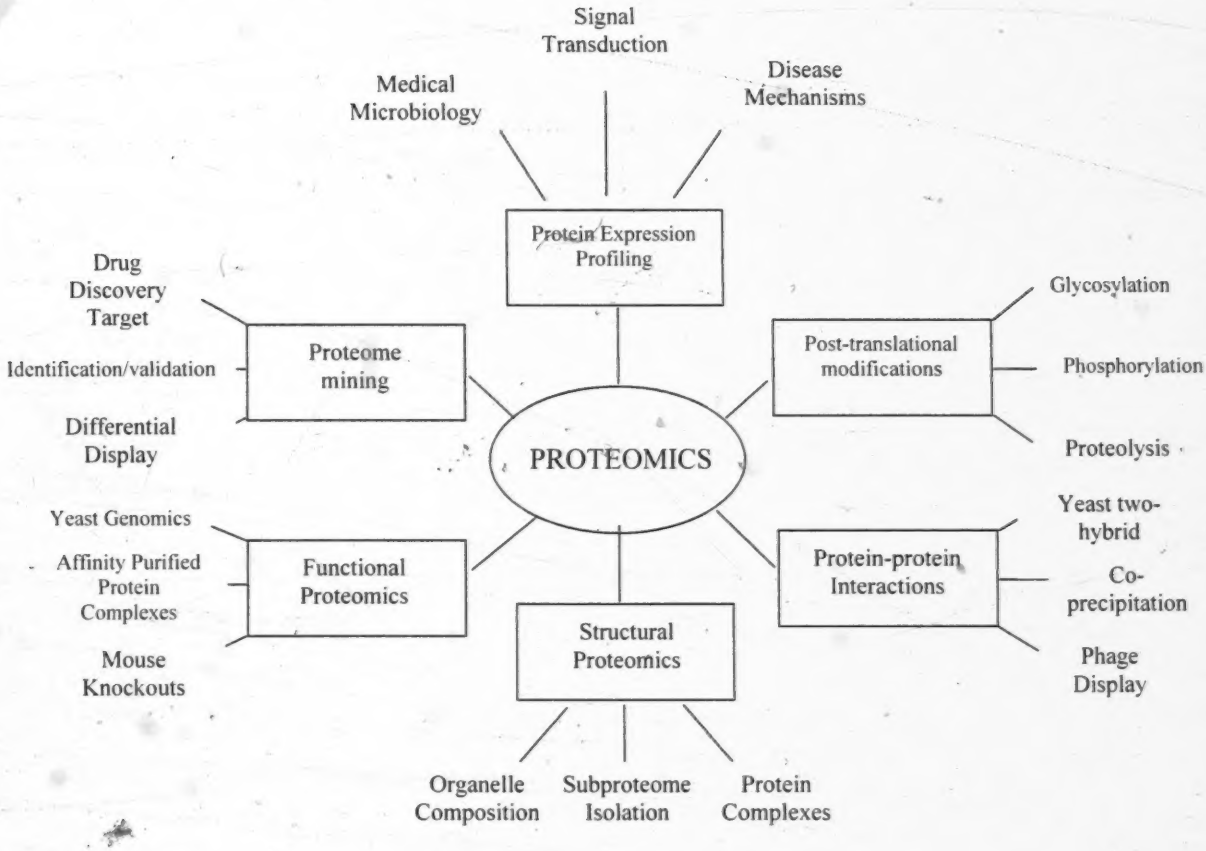


Figure 1.11. Different types of proteomics and their applications to biology (Graves and Haystead, 2002)

1.12 Two-Dimensional Gel Electrophoesis and Identification of Proteins

Although the development of two-dimensional electrophoresis, which is based on the separation of proteins first by their isoelectric point and second by their size, was a major accomplishment in 1975 (O'Farrell, 1975), the individual protein spots that showed up on the gels could not be identified easily. Only defined mutations could identify protein spots through comparisons of two-dimensional gels. For example, a protein spot may show up on the gel of one organism, whereas a mutant organism showing, or lacking a particular phenotype may not show that particular spot, indicating the protein in question maybe responsible for that particular known phenotype. This technique was long and tedious and not reliable. The first major technology to emerge for identifying unknown proteins was sequencing using Edman degradation (Edman, 1949), however the sensitivity of this technique was low at first and not very useful for identifying proteins in two-dimensional gels. A major accomplishment came about in the 1980's with the development of microsequencing techniques for electroblotted proteins which led to the creation of the first 2D gel databases (Aebersold, 1987). Improvements in microsequencing technology resulted in increased sensitivity of Edman sequencing to picomole amounts (Aebersole *et a.,l* 1987). One of the most important developments in protein identification has been the use of mass spectrometry (MS) technology. In the last decade, the sensitivity of analysis and accuracy of protein identification has increased by several orders of magnitude (Graves and Haystead 2002; Anderson, 2000). Proteins in the femtomolar range now can be identified from gels because MS is so sensitive. MS

sensitivity is also evident in the fact it can lead to the identification of proteins in mixtures.

The recent rapid growth in proteomics has been a direct result of advances made in large-scale nucleotide sequencing. Since the genomes of so many organisms are now sequenced or are in the process of being sequenced, protein peptides identified through MS analysis can be compared to either the genome of the organism under study or an array of microbial genomes. Without the sequenced genomes of so many organisms, proteins could not be identified despite the improvements made in MS.

1.13 Strains UWD, UWDS, 113, 113S

A. vinelandii strains used in this study included UWD, UWDS, 113 and 113S. Strain UWD, whose parent strains are OP and 113, is a PHB over-producer, is capsule negative and does not form cysts. AlgU, an alternative sigma factor responsible for alginate formation in exponential phase, is inactive in UWD due to the presence of an insertion element in the *algU* gene (Tindale *et al.*, 2000). Strain UWD has the ability to form alginate only in stationary phase when alginate biosynthesis is independent of AlgU, but proposed to be dependent on RpoS (Tindale, 2000). *A. vinelandii* strain 113 is also a PHB over-producer coming from the parental strain 12837. This strain does not have an insertion element in AlgU and can therefore produce alginate in exponential phase and stationary phase. Strains UWDS and 113S are the subsequent *rpoS* mutant strains of UWD and 113 (S. Kujat-Choy, unpublished). By inserting a kanamycin resistance cassette into the *rpoS* gene of *A. vinelandii*, strains UWDS and 113S were

created from the parent strains UWD and 113, respectively. Figure 1.12 shows the lineage of the strains discussed above.

1.14 Thesis objectives

In the first part of this thesis the production of cell protein, PHB, alginate and alkylresorcinols in *A. vinelandii* RpoS mutant strains (113S and UWDS) and *A. vinelandii* wild-type strains (113 and UWD) was measured. The goal of this work was to find out if the RpoS mutant strains were affected in their production of these secondary metabolites. Also studied was viability of UWD compared to UWDS after cells were subjected to a stress such as; stationary phase, oxidative stress or osmotic stress.

The aim of the second part of this thesis was to examine the effects RpoS has on *A. vinelandii* using a proteomic approach. More specifically the goal was to identify proteins turned on or up-regulated by this sigma factor during times of stress, in this case the stress being late stationary phase. In bacteria such as *E. coli* and *Salmonella* spp., much work has been done finding out how RpoS itself is regulated; it is likely that the regulatory network in *A. vinelandii* functions in a similar manner. In comparison however, little is known about the regulatory network over which RpoS exerts control except that the expression of RpoS is essential for optimal survival. The work presented in this thesis was undertaken in hopes of finding out the role RpoS plays in inducing expression of genes essential for stress survival in *A. vinelandii*. Also, because of the difficulties in obtaining good, reproducible results using a two-dimensional electrophoresis approach in any organism, the methods and results from this study

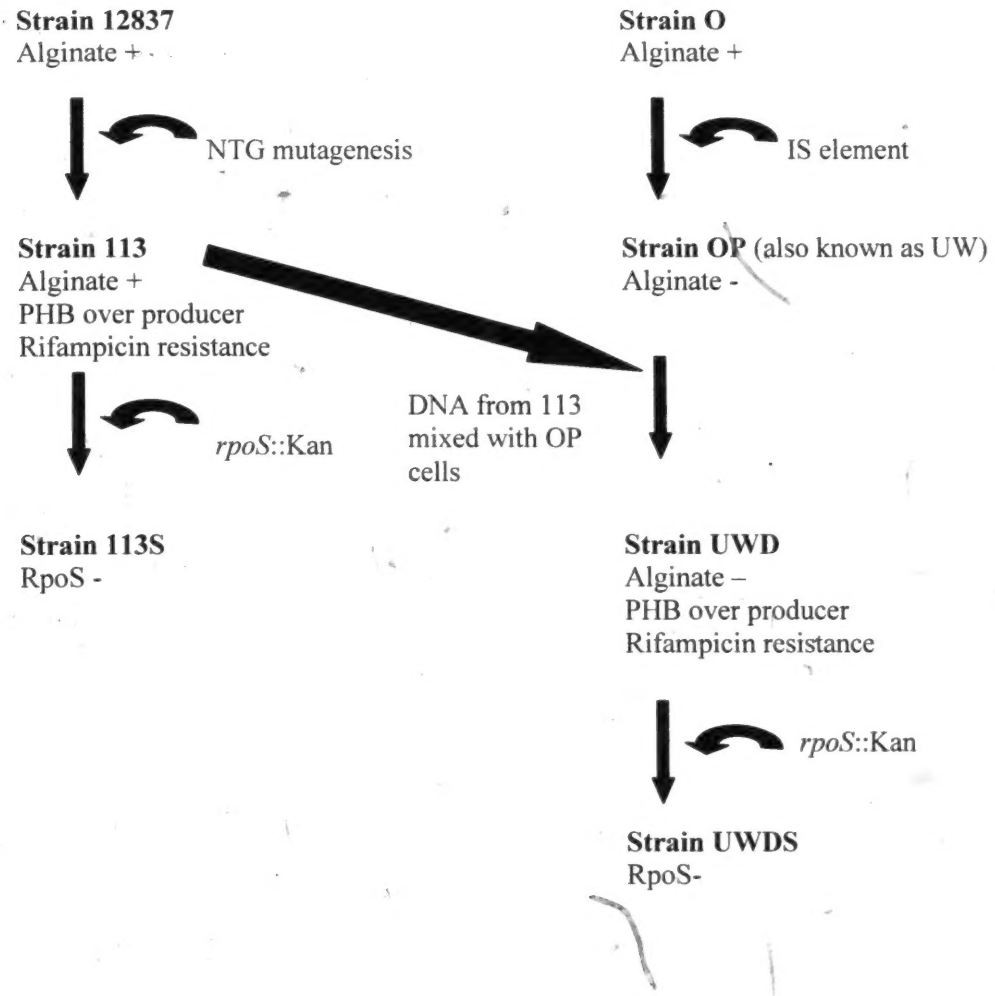


Figure 1.12. Lineage of *A. vinelandii* strains UWD, 113, UWDS and 113S. Strain UWD was formed by mixing DNA from 113 cells with UW cells. Through natural competence, strain UW took up this DNA and natural recombination occurred forming strain UWD (Page and Knosp, 1989).

provide invaluable information for future two-dimensional protein work using the model organism *A. vinelandii*.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial Strains

Azotobacter vinelandii strains UWD and 113 and the *rpoS* mutant strains UWDS and 113S were used in the growth studies and stress studies done in the first part of this work. In the second part of this work, the proteomic studies, strains UWD and UWDS were used. RpoS mutants were created by Sonya Kujat-Choy by inserting a kanamycin resistant cassette into a unique Nru1 site within the *rpoS* gene carried on a plasmid. The plasmid was confirmed through restriction digests and sequencing. Since *A. vinelandii* is naturally competent when iron limited, the plasmid was easily taken up and chromosomal gene replacement through homologous recombination produced the kanamycin resistant strains UWDS and 113S.

2.2 Growth studies

2.2.2 Growth Conditions

A. vinelandii strains were kept on slants made up of 1.5% to 1.8% agar with Burk's Buffer (BB: 0.81 mM $MgSO_4$, 0.58 mM $CaSO_4$, 5 μM Na_2MoO_4, and 12.5 μM $FeSO_4 \cdot 7H_2O$ in 5 mM potassium phosphate buffer, pH 7.2), 1% w/v glucose (G) as a carbon source and 0.11% ammonium acetate as a fixed source of nitrogen (N). *A. vinelandii* was grown aerobically on slants at 28-30 °C and the slants were stored indefinitely at room temperature. Cells for growth studies, stress survival studies and protein harvesting were grown in liquid culture at 28 to 30 °C shaking at 200 rpm. Liquid cultures consisted of 100 ml BBGN in a 500 ml flask. Cultures were started by

washing cells off slants using 1-3 ml sterile BB or sterile milli-Q H_2O. Cells for viable counts were grown on plates containing 1.5% agar made with BBG and no nitrogen source. Kanamycin was added to the medium as required to a final concentration of 10 μg/ml on solid media as well as in liquid media.

2.2.3 Lowry Method for Quantification of Total Protein

The first step in the Lowry (Lowry *et al.*, 1951) method for determination of total protein involved the digestion of cell pellets at 80°C for 1 hour in 0.1 M NaOH. Samples containing up to 100 μg of protein in a volume of 500 μl were mixed with 2.5 ml of reagent A (1.0 ml of 1% w/v sodium potassium tartrate, 1.0 ml of 0.5% w/v $CuSO_4$ in 50 ml of 2% w/v Na_2CO_3 in 0.1 M NaOH). The mixture was then incubated for 10 min at room temperature after which 250 μl reagent B [1 volume distilled H_2O, 1 volume Folin & Ciocalteu's Phenol reagent (Sigma)] was added. Following incubation for 30 min at room temperature, the absorbance of each sample was read at 620 nm and the protein concentrations were compared to a standard curve of known concentrations of bovine serum albumin (BSA) between 1 and 100 μg.

2.2.4 PHB Dry Weight Determination

For PHB dry weight determination, all centrifugation steps were done in a clinical centrifuge on setting number 5 (5 000 x g). The first step in the procedure (Law and Slepecky, 1961) involved dispensing 10 ml of culture fluid into clinical centrifuge tubes

followed by a 15 min centrifugation step. The supernatant was discarded and 10 ml of undiluted commercial bleach was added to the pellet. The mixture was incubated either overnight at room temperature, or for 1 hr at 45 °C. The suspension was then centrifuged for 10 min and the supernatant was again discarded. Pellets were washed with 5 ml of distilled water, transferred to a 13 mm test tube and centrifuged for another 10 min. Supernatant was discarded and the pellet was resuspended in 3 ml of acetone. This mixture was then transferred into pre-weighed dry weight pans that had previously been baked at 180 °C for 10 min to remove any moisture that may affect weighing. The weighing pans and the acetone/PHB mixture were then placed in the fume hood to allow the acetone to evaporate. Once there were no traces of acetone, the baking pans were weighed again and the weight of the PHB was recorded.

2.2.5 Fast Blue B Assay for Alkylresorcinol

To determine the amount of alkyresorcinols in *A. vinelandii* cells, the Fast Blue B Alkylresorcinol assay was used (Teluscik *et al.*, 1981). Cells were pelleted from 5 ml of culture and supernatant was discarded. To the pellet, 5 ml of acetone was added, and the pellet was resuspended by vortexing. Alkylresorcinols were then allowed to extract overnight. A total of 100 µl of the extract was then transferred to a new tube and 40 µl of acetone was added. The reagent mix was made by adding 1 part Fast Blue B stock (0.05% w/v Fast Blue salt in acetic acid) to 4 parts n-propanol and 2 ml of this was added to the acetone and extract mixture. Following incubation for 60 min at room temperature in the dark, the absorbance of each sample was read at 520 nm and the alkylresorcinol

concentrations were compared to a standard curve of known concentrations of orcinol between 0 and 7 μg.

2.2.6 Carbazole Assay for Alginic Acid

To determine the amount of alginate in *A. vinelandii* cultures, the carbazole assay for alginic acid was used (Knutson and Jeanes, 1968). First, three volumes of cold 95% v/v ethanol was added to the cultures and they were left overnight at 4 °C. The solution was then filtered onto a GF/A glass fiber filter and the filter was washed with 20 ml of cold 95% v/v ethanol. The filters were dried and suspended in an appropriate volume of 0.1 M NaOH that was determined by the initial volume of supernatant used and/or the amount of alginate precipitated. In a 13 mm test tube 0.35 ml of the sample was mixed with 3 ml of a sulfuric acid-0.1 M borate solution (2.5 ml of 4 M borate in 8 M KOH was added to 100 ml concentrated sulfuric acid). Following incubation for 10 min at 100 °C, the absorbance of each sample was read at 520 nm and the alginate concentrations were compared to a standard curve of known concentrations of alginate between 0 and 60 μg.

2.3 Stress Survival Studies

To find the percent survival of UWD and UWDS during stationary phase, UWD and UWDS cells were grown in 100 ml liquid culture as described in Section 2.2.2 for 5 days. Viable count assays were done every 24 h, spreading dilutions of 10^{-2} to 10^{-8} in duplicate onto BBG plates. Colonies were counted after incubation at 30°C for 3 days.

Samples were also taken after 6 h and every 24 hours, in conjunction with viable count assays, for microscopic examination. To find the percent survival of UWD and UWDS after exposure to oxidative stress, hydrogen peroxide (50 mM) was added to 18 h cultures grown in liquid BBGN. Samples were taken at t=0 (before the addition of H_2O_2), and at t=10, 30, 60, 120 and 240 min (after the addition of H_2O_2). Each sample was diluted and spread in duplicate on BBG agar for determination of viable cell numbers after incubation at 30°C for three days. Cells exposed to osmotic stress were grown for 18 h in liquid BBGN, centrifuged and then re-suspended in BBGN medium containing 0.5 M NaCl. Samples were taken at t=0 (before re-suspending cells in media containing 0.5 M NaCl) and at t=10, 30, 60, 120 and 240 min (after re-suspending cells in media containing 0.5 M NaCl). Each sample was diluted as described above and spread in duplicate on BBG agar for determination of viable cell numbers after incubation at 30°C for three days.

2.4 Proteomic Studies

2.4.1 Preparation of protein for two-dimensional gels

Cells were grown in liquid medium for 48 hrs as described previously (Section 2.2.2) after which 10 ml of the culture was spun down for one minute in a clinical centrifuge. The resultant supernatant was discarded and the pellets were resuspended in 1 ml of two-dimensional lysis buffer [consisting of either 8M Urea, 4% w/v Triton® X-100, 2% v/v Pharmalyte pH 3-10 (Amersham Biosciences) made up in milli-Q-H_2O, or 7M urea, 2M thiourea, 4% w/v 3-[chloramidopropyl dimethylammonio] -1-propane-

sulfonate hydrate) (CHAPS), 60 mM dithiolthreitol (DTT) and 2% v/v Pharmalyte pH 3-10 also made up in milli-Q-H_2O] and placed in a new clean 1 ml Eppendorf tube. Cells were lysed using sonication on a Braun-Sonic 2000 apparatus using a standard probe set at 0.14 Watts in two 30 second intervals. Sonication was done directly into 1 ml of lysis buffer and the insoluble material was spun down in a micro-centrifuge at 13 000 x g for 1 min. The resulting cell free extract (CFX) was subsequently transferred to another tube and then "cleaned up" (see Sections 2.4.1 and 2.4.2) and quantified using either the Lowry assay (Lowry, 1951), the Bradford assay (BioRad) or the Amersham 2D quantification kit (assays described in Section 2.6.3). To prevent proteolysis, protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and pepstatin A were added to the extracts to a final concentration of 1 mM and 2 µg/ml, respectively. Samples were subsequently stored at –70 °C or used immediately.

2.5 Protein Clean-up

2.5.1 Protein precipitation and re-suspension

In an effort to rid the protein samples of salts, nucleic acids and other non-protein impurities affecting isoelectric focusing as well as to concentrate protein samples that were too dilute, proteins were precipitated from the CFX using acetone or trichloroacetic acid (TCA) or a combination of both. For acetone precipitation, three volumes of ice-cold acetone were added to the extract. Proteins were allowed to precipitate at –20°C for 2-3 h. Proteins were then pelleted by centrifugation at 13 000 x g for 1 min and all residual acetone was removed by pipetting and air-drying. For TCA precipitation, TCA

was added to the CFX to a final concentration of 20% v/v and proteins were allowed to precipitate on ice for 30 min. The suspension was then centrifuged and the pellets were washed with ethanol to remove residual TCA. When using a combination of TCA and acetone, 500 µl of the CFX was suspended in 1 ml 10% w/v TCA in acetone containing 20 mM DDT. Proteins were precipitated for 1 h at –20 °C. Proteins were then pelleted by centrifugation at 13 000 x g for 1 min and pellets were washed with cold acetone containing 20 mM DDT. Residual acetone was removed by air-drying the pellet.

2.5.2 PlusOne 2-D Clean-Up Kit

An alternative method for ridding the protein samples of contaminants was to use the PlusOne 2-D Clean-Up Kit from Amersham Biosciences. In the first step of this procedure, three volumes of precipitant (supplied with the kit) were added to one volume of CFX. Samples were mixed by vortexing briefly and incubated on ice for 15 min. This step was repeated using co-precipitant (supplied with the kit) and the sample was mixed by vortexing. The tubes were centrifuged at 8 000 x g for 10 minutes. Immediately after centrifugation as much supernatant as possible was removed by careful pipetting, so as not to disturb the pellet. This step was repeated to bring any remaining liquid to the bottom of the tubes and all remaining supernatant was removed. Enough milli-Q-H_2O was added to just cover the pellet and the tubes were vortexed to disperse the pellet. Wash buffer (supplied with the kit) was pre-chilled for at least 1 h at –20 °C and 1 ml was added to the precipitated protein for each original volume of sample. To the same solution, 5 µl wash additive (supplied with the kit) was added to each tube, regardless the

volume of original sample. The suspension was then vortexed until the pellet was fully dispersed. The suspension was incubated at -20 °C for 30 min, vortexing every 10 min. The tubes were then centrifuged again at 8 000 x g for 10 min and the supernatant was discarded. The resulting pellet was allowed to air dry for 5 min after which it was resuspended in 500 µl rehydration solution for first dimension IEF (8 M Urea, 2% w/v CHAPS or Triton® X-100, 2% v/v IPG Buffer, 0.002% w/v bromophenol blue and 20 mM DDT added just before use).

2.6 Quantification of Protein for two-dimensional analysis

2.6.1 Lowry Method for Quantification of proteins for two-dimensional analysis

The Lowry method for quantification of proteins in CFX for two-dimensional analysis is the same as described in Section 2.3 except the first step of digestion in 0.1 M NaOH for 1 hr at 80 °C is omitted. CFX prepared by sonication was diluted 1/100 in dH_2O before quantifying.

2.6.2 Bradford Protein assay

The Bradford dye reagent [100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, 100 ml 85% w/v phosphoric acid and dH_2O (Bio-Rad)] was first diluted 1:4 in dH_2O after which it was filtered through a Whatman #1 filter. This diluted reagent was used immediately or kept in the fridge a maximum of two weeks. Protein sample (100 µl previously diluted 1/20 in dH_2O) was treated with 5 ml of the Bradford dye reagent,

vortexed, and incubated at room temperature for at least 5 min. Following incubation, the absorbance of each sample was read at 595 nm and the protein concentrations were compared to a standard curve of known concentrations of BSA between 0 and 900 µg.

2.6.3 PlusOne 2-D Quant Kit

The PlusOne 2-D Quant Kit (Amersham Biosciences) is a method for quantifying proteins for two-dimensional electrophoresis that is able to tolerate the presence of detergent (i.e. CHAPS or Triton® X-100) and reductants (i.e. DDT) in the protein samples better than other protein assays. The first step in this procedure was to add 10 µl of protein to be assayed to separate Eppendorf tubes. To each tube, 500 µl of precipitant (supplied with kit) was added, vortexed and incubated at room temperature for 2-3 min. In the next step, 500 µl of co-precipitant (supplied with kit) was added to each tube and the tubes were mixed gently by inversion. All tubes were then centrifuged at 10 000 x g for 5 min in order to sediment the protein. The supernatant was then decanted from the small pellet visible on the side of the tube. The tubes were spun again briefly in order to facilitate removal of all traces of remaining supernatant. To each tube, 100 µl copper solution (supplied with kit) and 400 µl of milli-Q-H_2O was added and the tubes were vortexed. In the next step, 1 ml of working colour reagent (100 parts colour reagent A mixed with one part colour reagent B, supplied with the kit) was added and instantaneous mixing was ensured by introducing the reagent as rapidly as possible. Following incubation for 20 min at room temperature, the absorbance of each sample was read at

480 nm and the concentrations were compared to a standard curve of known concentration of BSA between 1 and 100 µg.

2.7 Gel Electrophoresis

2.7.1 First Dimension: IPGphor strips

To run *A. vinelandii* total protein in the first-dimension, Immobiline DryStrip pH 3-10 (Amersham) immobilized gradient strips were used. To each strip either 15 µg of total protein (for silver staining) or 200-250 µg total protein was added (for Sypro® Ruby and Coomassie staining) in a total volume of 250 µl of re-hydration solution (8M Urea, 2% v/v CHAPS or Triton X-100, 2% v/v IPG Buffer, 0.002% w/v bromophenol blue, and 20 mM DDT added just before use). The strips were rehydrated in the sample solution for 12 h after which isoelectric focusing on an IPGphor Isoelectric Focusing Unit (Amersham Biosciences) took place. Isoelectric focusing began by first subjecting the strips to 500 V for 1 h, immediately followed by 1000 V for 1 h and finally 8000 V for 2 h. After focusing, strips were equilibrated in SDS equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M Urea, 30% w/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue in dH_2O. Just before use 100 mg DTT was added per 10 mL SDS equilibration buffer.) This was done in a screw cap tube, gently shaking with the support film of the strip toward the wall for 15 min. Equilibrated strips were used immediately or stored at –80 °C.

2.7.2 Second Dimension: SDS polyacrylamide gel electrophoresis (SDS-PAGE)

A large format protein apparatus (Hoefer Scientific Instruments) was used to run the second dimension. Two 10% poly-acrylamide gels were prepared as follows:

Acrylamide: bisacrylamide solution 40% (37.5:1)	25 ml
1M Tris-HCl pH 8.8	37.5 ml
10% w/v SDS	1 ml
1.5 % w/v Ammonium persulfate	5 ml
TEMED	50 µl
Milli-Q-H_2O	31.5 ml

To seal the previously focused and equilibrated Immobiline Dry Strip in place, a 1% agarose sealing solution [100 ml SDS electrophoresis buffer (see below), 0.5 g agarose, 200 µL bromophenol blue stock solution (100 mg bromophenol blue, 60 mg Tris-base, dH_2O to 10 ml)] was used by pipetting it over top of the strip which was already placed on top of the acrylamide gel, ensuring that there were no air bubbles between the strip and the polyacrylamide gel. The proteins in the strip were then separated on the SDS-PAGE gel using SDS electrophoresis buffer (25 mM Tris-base, 192 mM glycine, 0.1% w/v SDS in 10 l dH_2O). The gels were run at 15 mA per gel for 15 min followed by 30 mA per gel for 5 h.

2.8 Staining of two-dimensional gels

2.8.1 Coomassie Colloidal stain

The Coomassie G-250 colloid stain (Bio-Rad) was made by dissolving 60 mg of Coomassie G250 in ethanol for 20 min. While the stain was dissolving, 100 ml of 85% phosphoric acid was added to 800 ml of water. The Coomassie G250 solution was then filtered into the phosphoric acid solution, stirring constantly. The filter was rinsed with 50 ml dH_2O and the resulting Coomassie G250 solution was either used immediately, or stored at room temperature.

To stain an SDS gel after electrophoresis, the gel was first rinsed in dH_2O and then fixed in a solution containing 40% v/v methanol, 10% v/v glacial acetic acid and 2.5% v/v glycerol for 15 min. The gel was then rinsed with dH_2O and placed in the Coomassie G250 solution for 1 hr. Finally, if greater sensitivity was required, the gel was destained in a solution of 30% v/v methanol and 3% v/v glycerol.

2.8.2 Silver stain

Before silver staining, all glassware was washed thoroughly with a dilute solution of nitric acid followed by rinsing three times with milli-Q-H_2O. During the silver staining procedure, gloves were worn at all times. Immediately after the electrophoresis run the gel was placed in 400 ml of a fixative solution containing 40% v/v methanol and 10% v/v acetic acid for 30 min. Following the first fixing step was another fixing step for 15 min in 400 ml of 10% v/v ethanol and 5% v/v acetic acid v/v. A third and final

fixative step was done with another 400 ml of fresh 10% v/v ethanol and 5% v/v acetic acid for 15 min. After the three fixing steps the gels were stained and developed. To make up the staining solution the following reagents were added to 35 ml of milli-Q-H_2O in a 250 ml Erlenmeyer flask; 5 ml silver complex solution (2% w/v silver nitrate and 2% w/v ammonium nitrate), 5 ml reduction moderator solution (10% w/v tungstosilic acid) and 5 ml image development reagent (2.8% v/v formaldehyde). The solution was stirred constantly with a Teflon coated stirring bar and used within 5 min. Immediately after the staining solution was fully mixed, 50 ml of room temperature development accelerator solution (5% w/v sodium carbonate) was added, stirring constantly. The staining solution was added to a dish and the gels were stained with gentle agitation for approximately 20 min or until brown protein spots appeared on the gel. Just before the desired colour intensity was obtained, the staining solution was siphoned off and 5% acetic acid was added to the gels to stop the reaction.

2.8.3 Destaining Silver Stained Gels

Silver stained gels were placed in a solution containing 0.4 g potassium ferricyanide in 200 ml of fresh sodium thiosulfate (0.2g/l) to destain. The gels were left in this solution until spots appeared, with the proteins in the gel destaining first, leaving the background dark. Eventually, if the gels were left in this solution long enough, the proteins and the background would both destain.

2.8.4 Sypro® Ruby Stain

Before staining the protein gels with Sypro® Ruby (Molecular Probes), the gels were first fixed in a solution of 10% v/v methanol and 7% v/v acetic acid for 3 h. The gels were then washed for 10 min in milli-Q-dH_2O. This wash step was repeated three times. The Sypro® Ruby stain was then poured directly onto the gels to a total volume of 100 ml per gel. Staining was done in a polypropylene dish to ensure minimal absorption of the dye. The gels were left in the stain overnight. To reduce background fluorescence and to increase sensitivity, the gels were transferred to a clean dish and washed in a solution of 10% methanol and 7% acetic acid for 30 min. This wash step was repeated three times.

2.8.5 Viewing Sypro® ruby stained gels

Proteins stained with Sypro® ruby were visualized using a 300 nm UV transilluminator. In this case an AlphaImager™ 2200 by Alpha Innotech was used and the gel was analysed using Alpha Ease FC software provided with the transilluminator. Before scanning, the surface of the UV transilluminator was cleaned thoroughly with milli-Q-H_2O to prevent stains from accumulating on the glass and causing high background fluorescence.

2.8.6 Coomassie Blue Stain

Coomassie Brilliant Blue R-250 (Bio-Rad) was dissolved in a 5:4:1 solution of methanol, dH_2O and acetic acid. Before the gel was stained it was placed in a plastic container with 30 ml of fixing solution (5:4:1 solution of methanol, dH_2O and acetic acid), with slow agitation for 2 h on an orbital shaker. Once the gel was fixed, this solution was discarded and the gel was covered with the Coomassie Brilliant Blue staining solution and left to agitate gently for 4 h. The gel was rinsed with 50 ml of fixing solution for 2 min then placed in a destaining solution (7% v/v acetic acid, 5% v/v methanol in dH_2O) for 2 h after which fresh destain solution was added until the protein spots appeared on the gels. To help with the destaining, Kimwipes™ were added directly to the vessel containing the destain solution and the gels.

2.8.7 Coomassie brilliant blue stained gels: Scanning with Odyssey

Proteins in Coomassie Brilliant Blue stained gels can be visualized at a wavelength of 700 nm using an Odyssey™ Infrared Imaging System (LI-COR Biosciences). Odyssey™ software was used to analyse the two-dimensional gels after they were scanned.

2.9 Spot Selection and excision

After protein gels were scanned and analysed, spots were chosen based on their presence in the parent (UWD) cells and absence in the mutant (UWDS) cells. Spot excision was done using a razor blade. While wearing gloves the cut out spots were placed in labeled Eppendorf tubes containing 50 μL of milli-Q-H_2O and stored at –20 °C until analysis.

2.10 Mass Spectrometry

Excised spots were sent to IBD (Institute for Biomolecular Design, University of Alberta) where Mass Spectrometry analysis was performed. An automated in-gel tryptic digestion was performed on the excised spots (Section 2.8) on a Mass Prep Station (Micromass). The gel pieces were destained, reduced with DDT, alkylated with iodoacetamide and digested with trypsin (Promega Sequencing Grade Modified) and the resulting peptides were extracted from the gel and analysed via Liquid Chromatography, Mass spectrometry/mass spectrometry (LC/MS/MS). This was performed on a CapLC (Waters) capillary HPLC and a Q-ToF-2 (Micromass) mass spectrometer using a Picofrit C18 reversed-phase capillary column (New Objectives). The extract was run down a capillary HPLC reversed-phase column at a flow rate of about 200-400 nanolitres per minute using a water/acetonitrile gradient with 0.2% v/v formic acid. At the tip of the capillary column was fused silica which has a voltage applied to it so the separated eluting peptides were ionized using electrospray ionization and the subsequent eluent was

introduced directly to the mass spectrometer. The Q-ToF detects a peptide, ignoring all other peptides with different molecular weights and fragments it in a collision cell using argon gas. The fragmentation occurs somewhat preferentially at peptide bonds giving a predictable MS/MS fragmentation pattern and the associated mass of the intact peptide.

2.11 Peptide analysis

Protein identification from the generated MS/MS data was done searching the National Center of Biotecnology Information (NCBI) non-redundant database using Mascot Daemon (Matrix Sciences). The data being analysed was the parent mass of the intact peptide and the associated fragment pattern. This allows for identification of the sequence that would likely generate such a fragmentation pattern. Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide. Once peptides were identified with a high confidence level they were searched against the recently sequenced *A. vinelandii* genome using the tBLASTn function on the webpage (**www.ava.biosci.arizona.edu/index.html**, accessed May 2003-September 2003).

CHAPTER 3: RESULTS

3.1 Growth Studies

3.1.1 Protein, PHB, Alginate and Alkylresorcinol in UWD and UWDS

In order to determine if the previously constructed RpoS mutant strain UWDS was deficient in producing protein, PHB, alginate or alkylresorcinols, its production of these compounds was compared to the parent strain, UWD. Total amount of protein produced in UWD and UWDS was determined using the Lowry assay (Lowry, 1951) was done. Samples were taken every 24 h and assayed accordingly. PHB production in both strains was also compared by determining the total dry weight of 10 ml of culture every 24 h. Comparison of the production of alginate and alkylresorcinols by the two strains, was carried out using the Carbazole assay and the Fast Blue B assay respectively, again harvesting every 24 h. The results of these assays are shown in Figure 3.1. Less protein, PHB and alginate were produced by strain UWDS compared to UWD. Strain UWD produced up to 0.93 mg protein per ml of cell culture whereas UWDS produced a maximum of 0.83 mg protein per ml of cell culture. In terms of PHB production UWD produced a maximum of 0.27 mg PHB per mg cell protein whereas UWDS produced a maximum of 0.20 mg PHB per mg cell protein. Strain UWD produced up to 0.77 mg alginate per mg protein and UWDS produced a maximum of 0.16 mg alginate per mg cell protein. No alkylresorcinols were produced by UWDS at all, whereas UWD produced up to 0.025 mg/mg of cell protein.

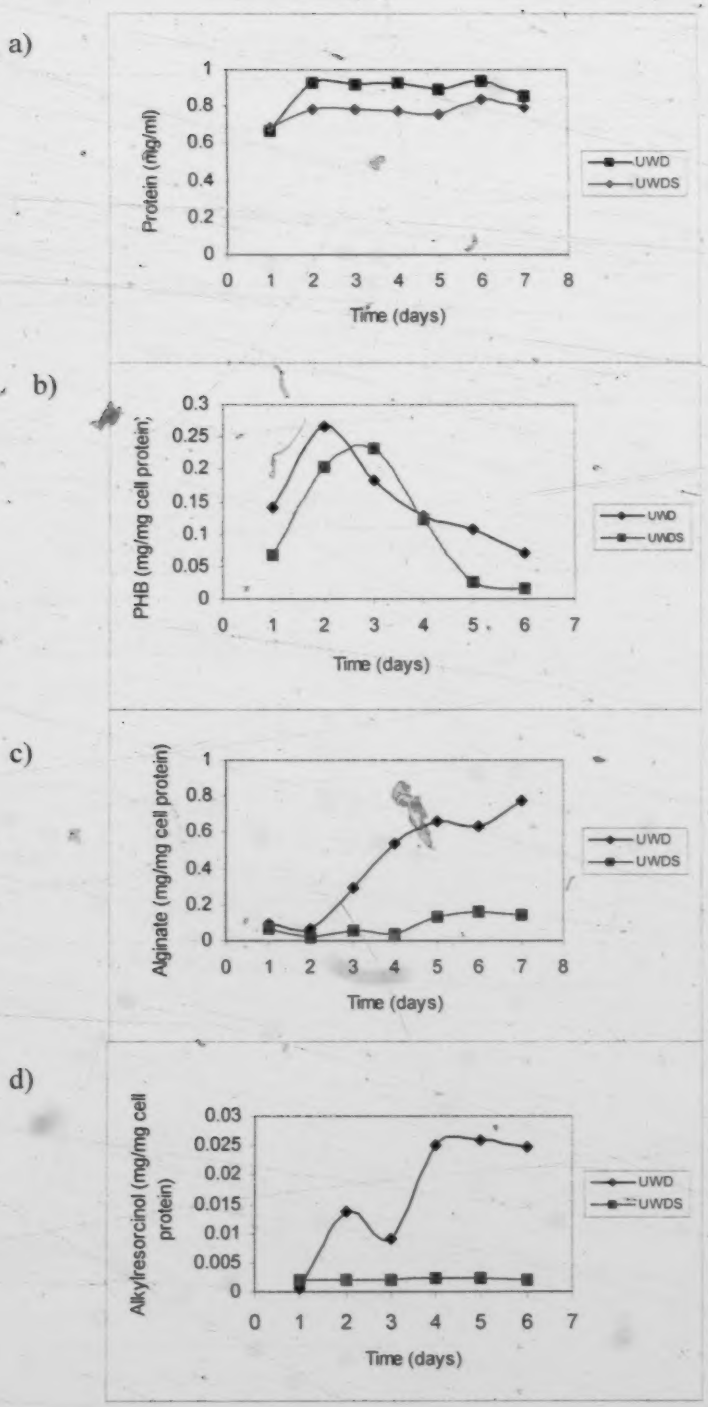
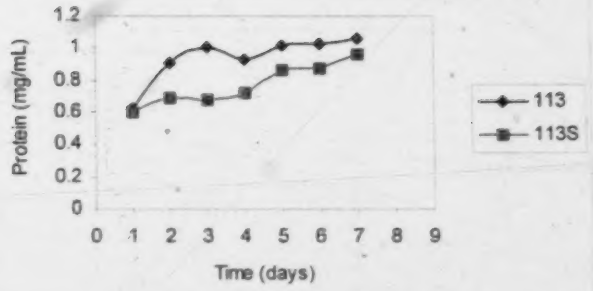


Figure 3.1 Results from growth study comparing *A. vinelandii* strains UWD and UWDS in their production of protein, PHB, alginate and alkylresorcinol. a) protein, b) PHB, c) alginate and d) alkylresorcinol. Samples were taken in duplicate and in all cases the range between the samples did not exceed the size of each point.

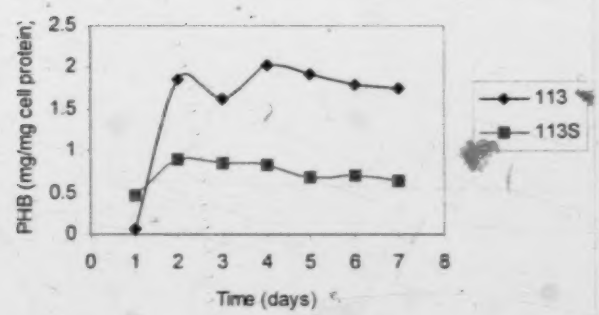
3.1.2 Protein, PHB, Alginate and Alkylresorcinol in 113 and 113S

In the same fashion as described above, protein, PHB, alginate and alkylresorcinols were measured over 7 days in strains 113 and 113S. Results similar to UWD and UWDS were found in all cases except for alginate. Alginate production was not as low in 113S compared to 113 as with UWDS compared to UWD. These results are shown in Figure 3.2. Strain 113S produced less protein compared to strain 113, with the maximum protein produced by 113S being 0.96 mg of protein per ml of cell culture and 113 being 1.06 mg of protein per ml of cell culture. In terms of PHB production, strain 113S produced a maximum of 0.009 mg of PHB per mg of cell protein and strain 113 produced 0.02 mg of PHB per mg of cell protein. Strain 113S produced up to 0.79 mg of alginate per mg of protein and 113 produced 1.03 mg of alginate per mg of protein. Again, alkylresorcinol production was non-existent in the RpoS mutant strain 113S whereas 113 produced up to 0.046 mg per mg of cell protein.

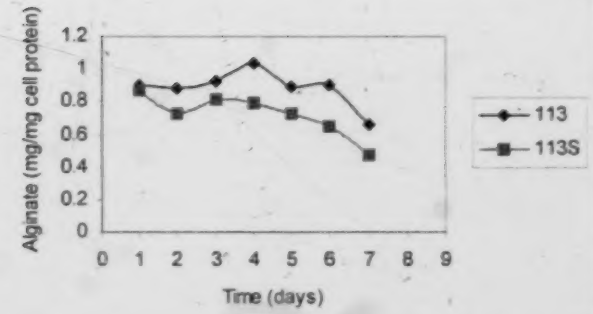
a)



b)



c)



d)

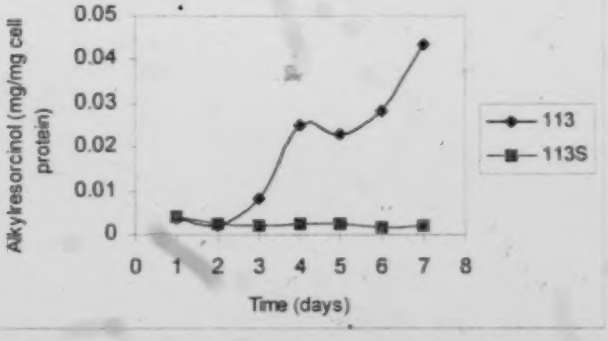
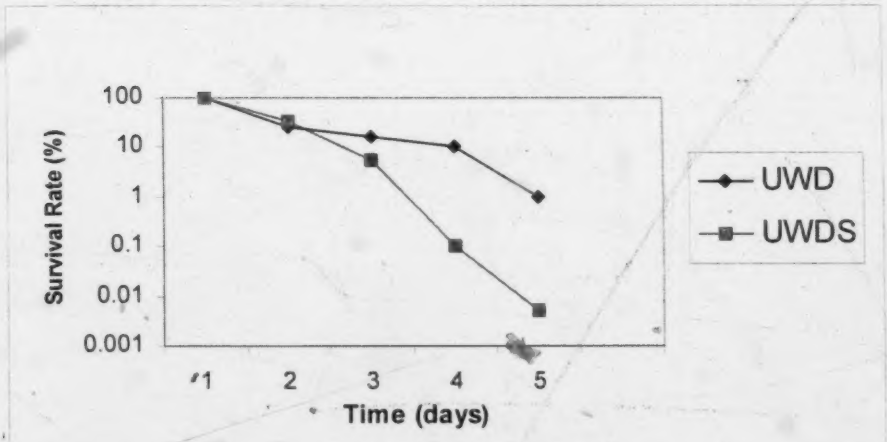


Figure 3.2 Results from growth study comparing *A. vinelandii* strains 113 and 113S in their production of protein, PHB and alkylresorcinols. a) protein, b) PHB, c) alginate and d) alkylresorcinol. Samples were taken in duplicate and in all cases the range between the samples did not exceed the size of each point.

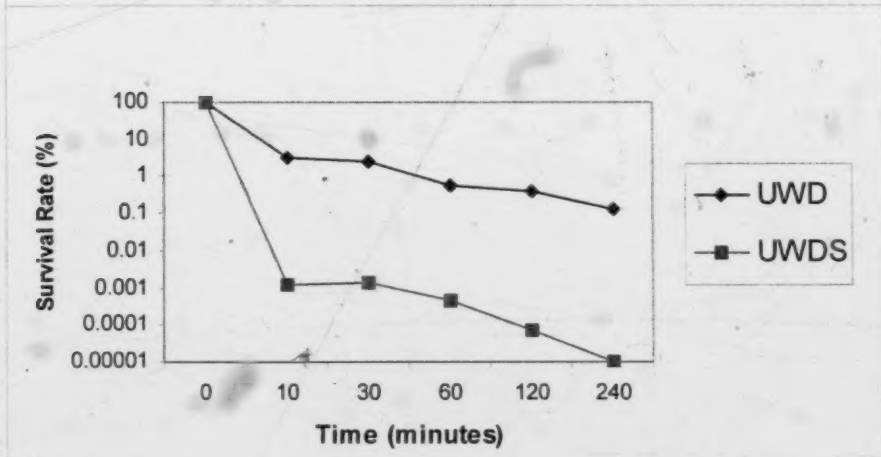
3.2 Stress Survival

The effect of the *rpoS* mutation on survival in stationary phase survival as well as after exposure to oxidative and osmotic stress was studied. This was done by determining the viable plate counts late into stationary phase and also after exposure of the cells to 50 mM hydrogen peroxide and 0.5 M sodium chloride. As shown in Figure 3.3, late into stationary phase the RpoS mutant strain UWDS had significantly decreased viability over time compared with the parent strain UWD. At t=1, after 24 hr of growth, viability for both UWD and UWDS was considered to be 100%. After 5 days however there was a 200-fold difference in survival with approximately 1% of UWD cells remaining viable, and only 0.005% of UWDS remaining viable. UWDS cells exhibited larger more ovoid or rod shaped cells compared to UWD during stationary phase with UWDS looking like exponential phase cells (i.e. smaller and more circular) late into stationary phase. The RpoS mutants exposed to oxidative stress also showed significant decreased viability over time compared to their parent strain. The results of the cells exposed to 50 mM hydrogen peroxide showed UWD having 0.1% viability after 240 min. and UWDS having only 1.0×10^{-5} % viability after 240 min. The RpoS mutant cells exposed to osmotic stress again showed a significant decrease in viability compared to their parental strain. After exposure to 0.5 M sodium chloride for 240 min, UWD had 0.05% remaining viable cells and UWDS had only 4.0×10^{-4} %. Graphs depicting these results are shown in Figure 3.3.

a)



b)



c)

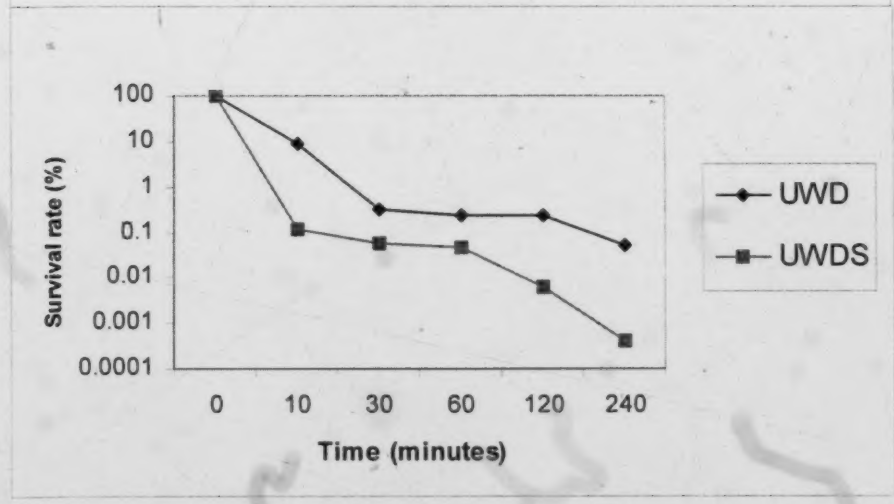


Figure 3.3. Results from stress study showing the viability of UWD and the *rpoS* mutant UWDS. One hundred percent survival corresponds to the viable cell count determined prior to exposure to the indicated stress. Survival on (a) entry into stationary phase, (b) exposure to 50 mM H_2O_2 and (c) exposure to 0.5 M NaCl.

3.3 Two-Dimensional Electrophoresis

3.3.1 Coomassie Colloidal and Silver Stained Gels

To find proteins under the control of RpoS, a two-dimensional electrophoresis approach was used to compare the protein profile of strain UWD to the protein profile of the RpoS mutant strain, UWDS. This was done by running total protein from these cells on IPGphor strips (Amersham Biosciences) having a pH range of 3-10. Proteins present on the UWD gel and not the UWDS gel were assumed to be under the control of RpoS, either directly or indirectly.

In the first attempt at running a two-dimensional gel, 200 μg of protein, quantified by diluting the sample and using a Lowry assay, was loaded onto the first dimension strip. The proteins were subsequently run in the first and second dimension and the resulting gel was stained using a Coomassie colloidal stain. According to the protocol, this method of staining should be sensitive enough to detect 200 μg of total protein per gel, however the results from this experiment showed otherwise, with no protein spots showing up on the gel. This experiment was repeated, this time adding 250 μg of protein instead of 200 μg however the results were the same with no protein showing up on the gels.

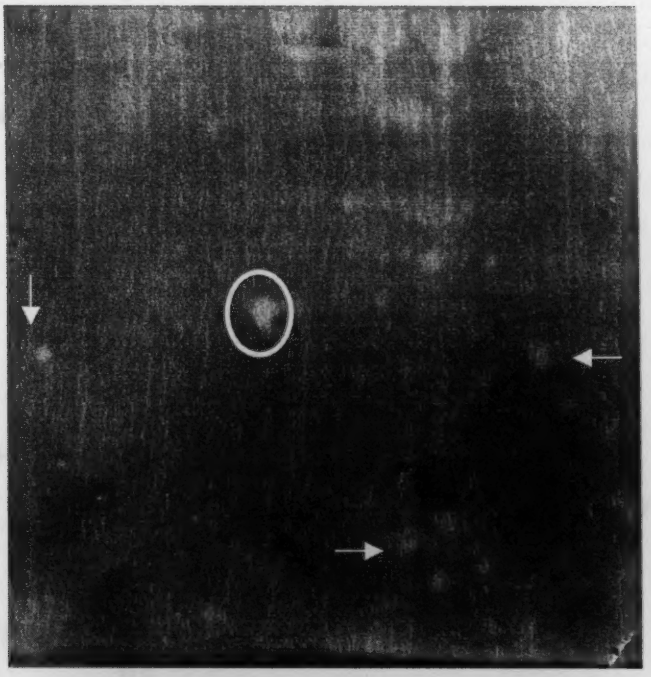
A more sensitive staining method, silver staining, was used as an alternative to the Coomassie colloidal stain. Silver staining is sensitive enough to detect protein levels as low as 8 μg per two-dimensional gel. Initially this method was not successful in detecting spots on the two dimensional gels with only horizontal and vertical streaks showing up on the gel. This streaking pattern is indicative of insoluble proteins, salts or nucleic acids in

the sample. To clean up the samples the proteins were precipitated using either acetone or TCA as well as a combination of both (described in the Chapter 2). In addition to cleaning the samples, the protein was also quantified in a different manner. The Bradford assay was used since it is more compatible with solutions containing detergents and reductants than the Lowry assay. After quantification, 15 μg of protein was added to the IPGphor strips and run in the first and second dimension. Proteins that had been precipitated with only acetone or only TCA revealed horizontal and vertical streaking on the silver stained gels. Proteins precipitated with a combination of both revealed no proteins at all. In this case, instead of discarding the gels, they were destained with the intention of trying to re-silver stain them and in doing this, spots showed up (Figure 3.4). These spots appeared as clear areas in a darker background and there was a definite similarity between the protein profiles of UWD and UWDS. One large spot appeared in the protein profile of UWD and not in UWDS indicating this protein was likely under the control of RpoS. This spot was excised and sequenced at IBD by MS analysis. The peptides generated matched a protein on the *A. vinelandii* genome showing homology to a uridine monophosphate kinase in *Methanothermobacter thermoautotrophicus*. The peptides generated as well as the protein sequence of the uridine monophosphate kinase are shown in Figure 3.5.

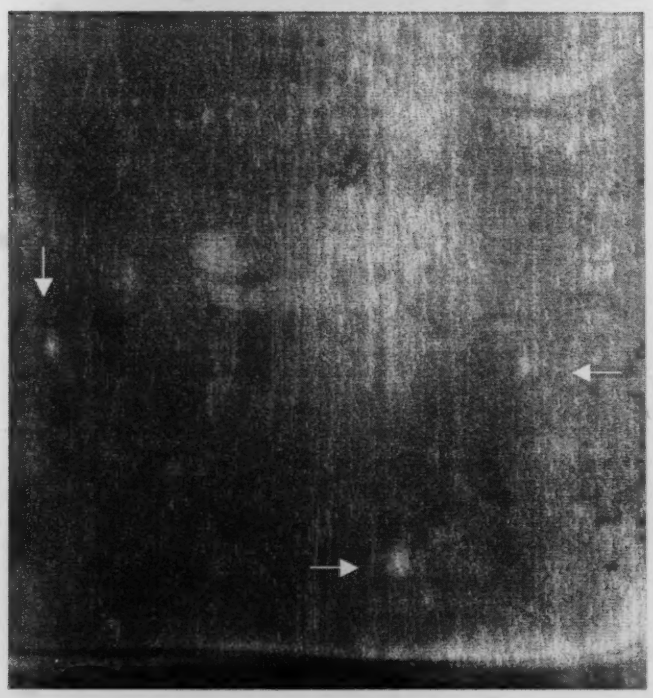
3.3.2 Sypro® Ruby Stained gels (First Set)

Although silver staining and subsequent destaining revealed several proteins on the two-dimensional gels, a more acceptable method of staining was necessary. In an

a)



b)



pH 7 3

Figure 3.4. Results of gels silver stained and destained. a) UWD two-dimensional protein profile b) UWDS two-dimensional protein profile. Proteins destained rapidly and appear as clear spots against a black background. Landmark proteins appearing on both gels are shown by the white arrows. The circled protein appears only in the wild type cells and not in the mutants. This protein was excised and sent for MS analysis.

MANSTAELEELLMQRSLTDPQLQAAAAAAADFRILPDATVIKIGGQSVIDR
GRAAVYPLVDEIVAARKNHKLLIGTGAGTRARHLYSIAAGLGLPAGVLAQ
LGSSVADQNAAMLGQLLAKHGIPVVGGAGLSAVPLSLAEVNAVVFSGMP
PYKLWMRPAAEGVIPPYRTDAGCFLLAEQFGCKQMIFVKDEDGLYTADP
KTSKDATFIPRISVDEMKAKGLHDSILEFPVLDLLQSAQHVREVQVVNGLV
PGNLTRALAGEHVGTIITAS

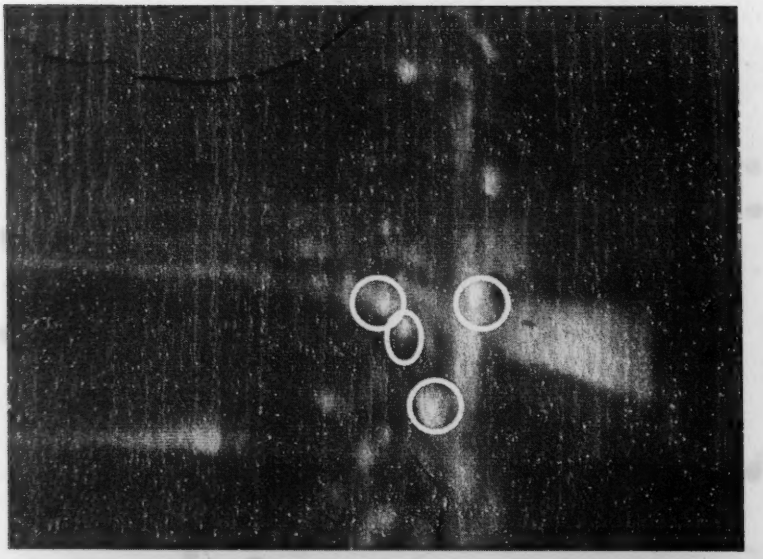
Figure 3.5. Amino acid sequence of the uridine monophosphate kinase protein identified from the silver stained and destained gel. Protein was digested using trypsin before analysis. Each highlighted area represents a peptide identified through MS.

attempt to achieve this a new stain, Sypro® Ruby was used. In addition to using this new stain, a new method of quantification using the PlusOne 2-D Quant Kit from Amersham Biosciences and a new method of cleaning up the protein samples using the PlusOne 2-D Clean-Up Kit was used. The results of the first set of Sypro® Ruby stained gels are shown in Figure 3.6. Using these new methods of cleaning, quantifying and staining, spots were observed in the protein profiles of UWD and UWDS. Although there were many spots, something that had not been observed up to this point, there was not a clear pattern between the gels and because of this, no protein spots were excised for MS analysis.

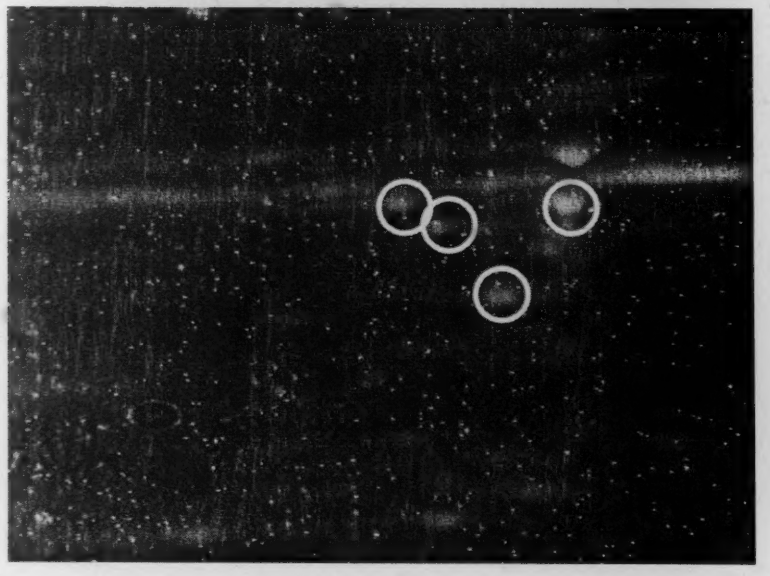
3.3.3 Sypro® Ruby Stained Gels (Second Set)

The second set of gels with protein prepared as in Section 3.2.2 and stained with Sypro® Ruby showed many distinct protein spots as shown in Figure 3.7. In an attempt to find and identify a landmark protein, a large spot appearing on both the UWD and UWDS gels was excised. This spot was sent to IBD for MS analysis. The peptides generated, when compared to the *A. vinelandii* genome indicated the protein was a para-crystalline surface layer protein showing high homology to its counterpart in *Aeromonas hydrophila*. The protein has a molecular weight of 60 kDa with a pI of 5.2. The protein sequence, as well as the peptides identified are shown in Figure 3.8.

a)



b)



pH 8 3

Figure 3.6. Results of first set of gels stained with Sypro® Ruby. a) UWD two-dimensional protein profile b) UWDS two dimensional protein profile. Proteins that are circled are landmarks that possibly may line up in both cell types.

a)

b)

pH 7 pH 3

Figure 3.7. Results of second set of gels stained with Sypro® Ruby. a) UWD two-dimensional protein profile. b) UWDS two-dimensional protein profile. Arrows are pointing to a protein in found in both UWD and UWDS that was identified through MS as having homology to the para-crystalline surface layer structure in *Aeromonas hydrophila.*

MMKKSLLALAVAALSANAFAFELGTDSALKYASEIKFDTTDGTELTLPAGET
VDVETGFSITAGNVRYVRFDITGAEFDTSNVAAADLVAAGLTIDTEYSVQVS
AVDSTNKKYVIFEITPLAAGGDMDNEQELNFSPKILAKSAANVTLQYRLYET
GVAALAGSDAVLASKSGSVISFAKALSFKATPNAAIGKIDVAQESKLFTTGNE
TDLGTVEVALDSAVLWTDGLATEAEDLLSDTANANSIVISGDFSSATDVQLA
GSSIDPDNLTATTATFPFANAASVIGSGPITFITNETDAISPSGFTTSLDLTAATN
SSATDISGGALGSLEKNGDSKEVNLSLKPKSEGGVYDNFVRISNTSSLAGKFFI
TVIADDGSTASLALSDVEGQPAELAARASTTQMRIDDIYAAAVAKGFALSGQ
GKLRLVVEGEVPSLDVQTYTVSKDANSFATF

Figure 3.8. Amino acid sequence of the para-crystalline surface layer protein identified from the second Sypro® Ruby stained gel. Protein was digested using trypsin before analysis. Each highlighted area represents a peptide identified through MS.

3.3.4 Coomassie Brilliant Blue Stained Gels

Staining with Sypro® Ruby is a fast and effective way to visualize proteins on a two-dimensional gel with the only downfall being the high cost ($60 per gel). When running many large format gels and using copious amounts of stain it is advantageous to find a more economical method of staining without loosing sensitivity. Fortunately this was achieved using Coomassie Brilliant Blue stain and subsequently scanning the gel at a wavelength of 700 nm using an Odyssey scanner (Li-Cor Biosciences). These gels are shown in Figures 3.9 and 3.10. Many distinct spots were visible on the UWD gel and not on the UWDS gel. Six of these spots were excised and sent to IBD for MS analysis.

3.4 Peptide Identification

3.4.1 Five Proteins Identified

Of the six protein spots chosen, five unique proteins were identified. These five proteins, along with their predicted molecular weight (calculated using GeneTools®) as well as their calculated molecular weight (calculated based on the distance the proteins travelled on the SDS PAGE gel) are summarized in Table 3.1.

3.4.2 Spot 1: Aconitate Hydratase 2

The peptides generated and identified from Spot 1 matched with a protein on the *A.vinelandii* genome showing homology (E value of 0.0) and 93% identity to aconitate

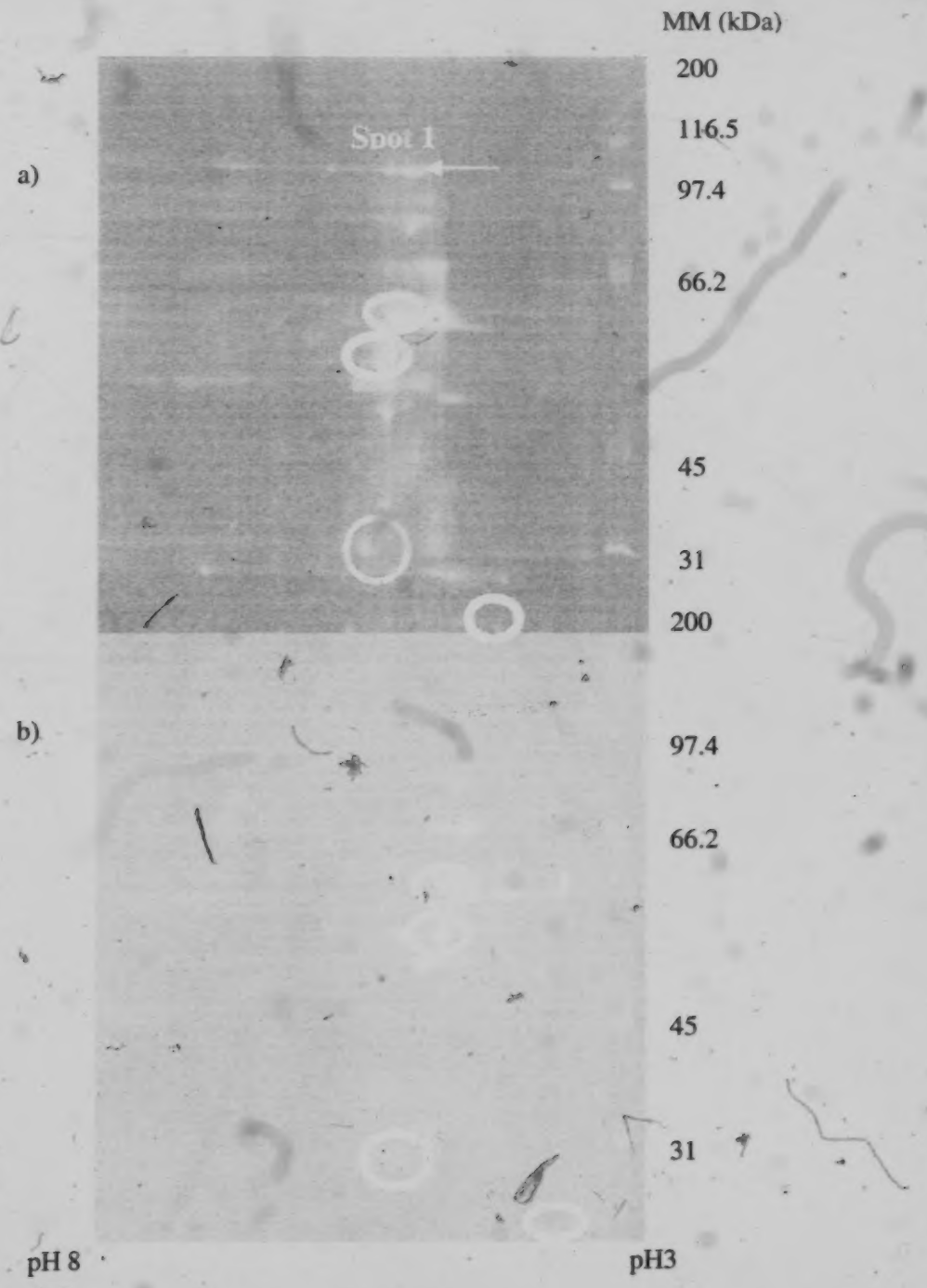


Figure 3.9. Results of gels stained with Coomassie Brilliant Blue a) UWD two-dimensional protein profile b) UWDS two-dimensional protein profile: Land-mark proteins are circled in white and the surface-layer protein previously identified is circled in yellow. The white arrow points to a protein found in UWD cells and not in UWDS.

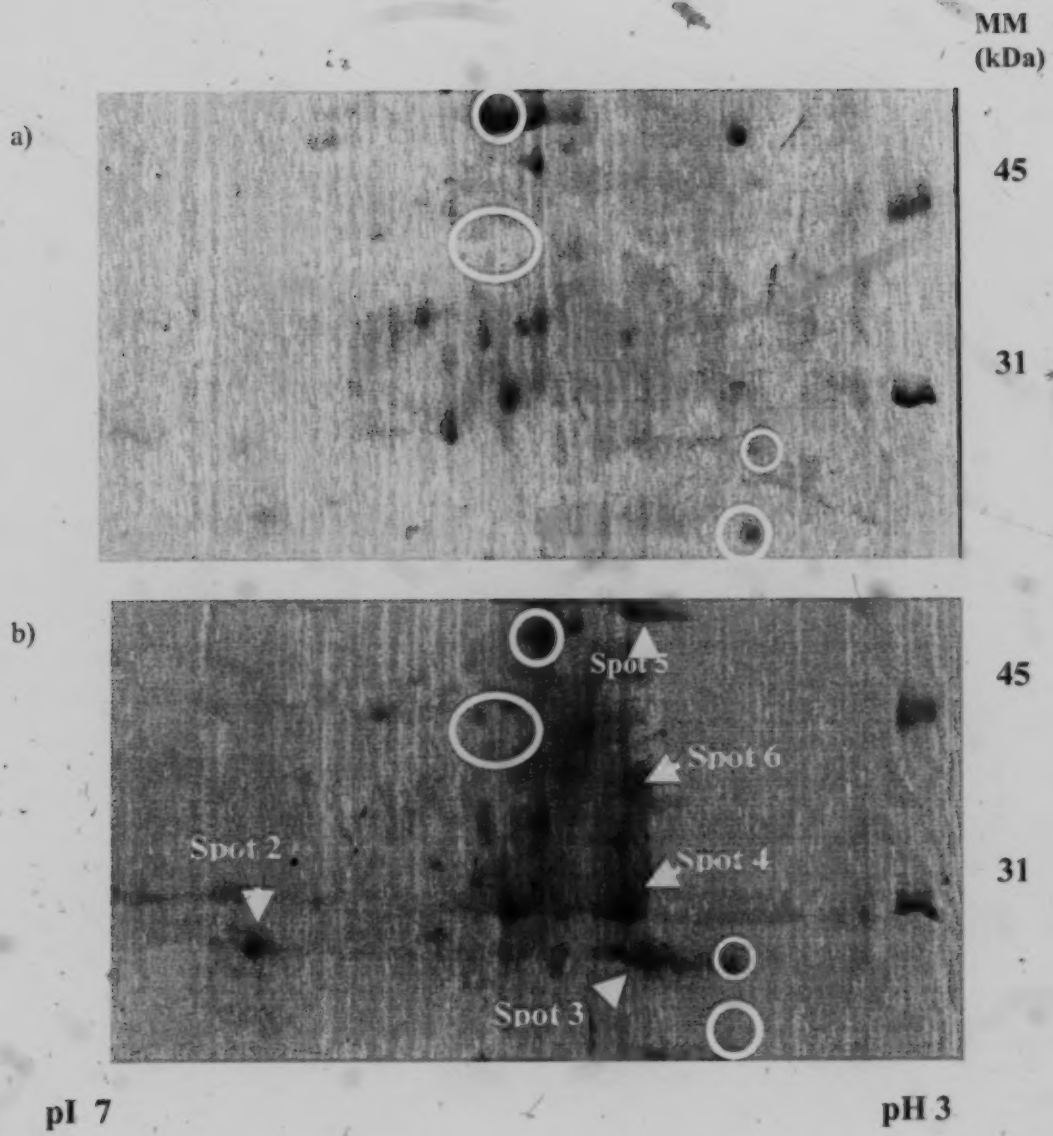


Figure 3.10. Close-up view of the two-dimensional gel stained with Coomassie Brilliant Blue from Figure 3.9. a) proteins from UWDS b) proteins from UWD. Land-mark proteins that appear on both gels are circled and proteins appearing only in the wild-type cells are indicated with the arrows.

Table 3.1: Proteins identified to be regulated by RpoS along with their predicted and calculated pI's and molecular weights.

Spot Number	Protein Homology (a)	Predicted pI from Peptools (b)	pI Read from gel	Predicted Molecular weight (kDa) (a)	Calculated Molecular weight (kDa) (c)
1	Aconitate Hydratase 2	6.2	5.5	91.5	97.7
2	UMPK (d)	6.6	6.25	28.3	25.5
3	UMPK (d)	6.6	5.1	28.3	25.5
4	UK(e)	6.6	5.1	28.3	25.5
5	OprF	5.4	4.9	37.6	46.7
6	NifU	5.9	5.0	33.3	32.3

(a) From *A. vinelandii* genome
(b) Based on *A. vinelandii* translated gene
(c) From SDS-PAGE gel (second dimension)
(d) Uridine monophosphate kinase
(e) Uridylate kinase

hydratase 2 of *P. aeruginosa* (GenBank # NC_002516). The molecular weight of the *A. vinelandii* protein (predicted by GeneTools) was 91.5 kDa with a pI of 6.2. According to the two dimensional gel results, the molecular weight was calculated to be 97.7 kDa and the pI 5.5. The sequence of this protein is shown in Figure 3.11, with the specific peptides generated from the tryptic digests and subsequent MS highlighted. The upstream and downstream regions of the gene coding for this protein are shown in Figure 3.12.

3.4.3 Spots 2 and 3: Uridine Monophosphate Kinase

Spot 2 and spot 3 both generated peptides corresponding to a protein on the *A. vinelandii* genome showing homology (E value of 1 x 10^{-7}) and 27% identity to a uridine monophosphate kinase in *Methanothermobacter thermoautotrophicus* (GeneBank # NC_000916). The peptides generated from the tryptic digestion of this protein and subsequently identified through MS are shown in Figures 3.13a and b.

VPQPLNAEQTAALVELLKNPPAGEEEFLLDLITNRVPPGVD
EAAYVKAGFLSAIAKGEAASPLIGKTRAIELLGTMQGGYNI
ATLVELLDSAELANAAAEQLKHTLLMFDAFHDVSERAKK
GNAAAKAVLQSWADGEWFTAKPAVPEKVTLTVFKVPGET
NTDDLSPAPDAWSRPDIPLHALAMLKMARDGIEPIQPGSV
GPLKQIEAVKAKGFPVAYVGDVVGTGSSRKSATNSVLWFF
GDDIPYVPNKRAGGFCFGTKIAPIFYNTMEDAGALPIEFDC
TNLAMGDVIDVYPVKGEVRRNGSDELVTTFALKTDVLLD
EVRAGGRIPLIIGRGLTEKAR[redacted]
[redacted]GYSLAQKMVGRACGLPEGKGVRPGTYCEPK[redacted]
[redacted]RDELKDLACLGFSADLVMQSFCHTAAYPKPIDV
KTHHTLPDFIMNRSGVSLRPGDGIIHSWLNR[redacted]
GGDSHTRFPIGISFPAGSGLVAFAAATGVMPLDMPESVLVR
FKGK[redacted]LVHAIPYYAIQAGLLTVEKKGKKNIFSG
RILEIEGLNDLTVEQAFELSDASAERSAAGCTIKLPEKAIAE
YLKSNITMLRWMIGEGYGDPRTLERRAQAMEAWLAKPQL
LEADKDAEYAAVIEIDLADVKEPVLCAPNDPDDARLLSSV
AGQKIDEVFIGSCMTNIGHFRAAGKLLDKVKGGIPTRLWL
APPTKMDAHQLTEEGYYGIYGKAGARMEMPGCSLCMGN
QARVQTGSTVVSTSTRNFPNRLGDATNVFLASAELASVASI
LGKLPTVEEYMEYAKNIDTMAADIYRYLSFDQIAEYKEAA
AKAELPVLQV

Figure 3.11. Amino acid sequence from the aconitate hydratase 2 protein identified from Spot 1 of the UWD two-dimensional gel. Protein was digested using trypsin before analysis. Each highlighted area represents a peptide identified through MS.

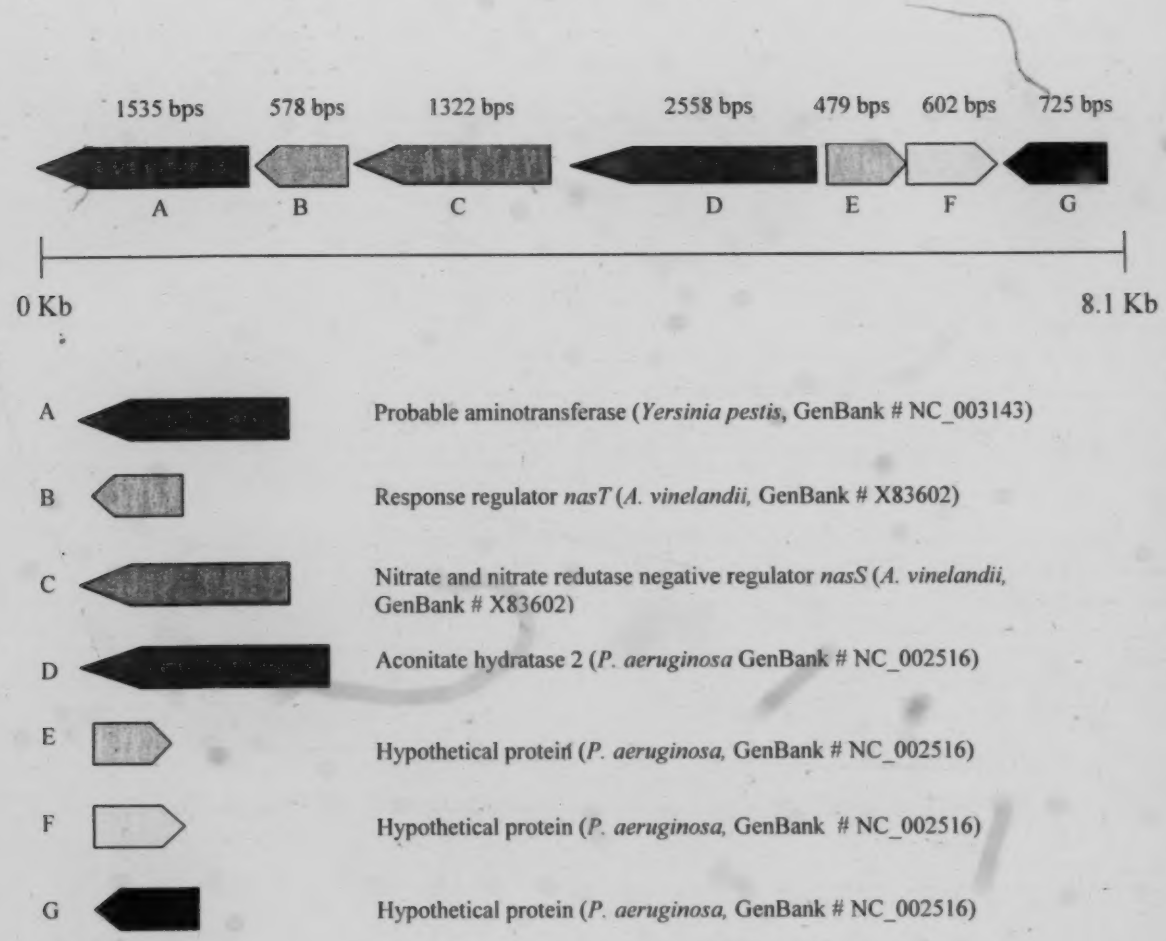


Figure 3.12. Organization of the region containing the gene encoding the aconitate hydratase 2 protein identified through two-dimensional electrophoresis to be regulated by RpoS in *A. vinelandii*. This fragment is found on contig 78 and spans nucleotides 62990 to 71858 on the *A. vinelandii* genome (www.azotobacter.org, 2003).

a)

MANSTAELEELLMQRSLTDPQLQAAAAAAADFRILPD
ATVIKIGGQSVIDRGRAAVYPLVDEIVAARKNHKLIG
IGAGTRARHLYSIAAGLGLPAGVLAQLGSSVADQNAA
MLGQLLAKHGIPVVGGAGLSAVPLSLAEVNAVVFSGM
PPYKLWMRPAAEGVIPPYRTDAGCFLLAEQFGCKQMI
FVK[redacted]TSKDATFIPRISVDEMKAKGLHDS
ILEFPVLDLLQSAQHVR[redacted]R[redacted]
[redacted]

b)

MANSTAELEELLMQRSLTDPQLQAAAAAAADFRILPD
ATVIKIGGQSVIDRGRAAVYPLVDEIVAARKNHKLIG
IGAGTRARHLYSIAAGLGLPAGVLAQLGSSVADQNAA
MLGQLLAKHGIPVVGGAGLSAVPLSLAEVNAVVFSGM
PPYKLWMRPAAEGVIPPYRTDAGCFLLAEQFGCKQMI
FVK[redacted]TSKDATFIPRISVDEMKAKGLHDS
ILEFPVLDLLQSAQHVR[redacted]R[redacted]
[redacted]

Figure 3.13. Amino acid sequence from the uridine monophosphate kinase protein identified from Spot 2 and Spot 3 of the UWD two-dimensional gel. a) amino acid sequence from Spot 2 with peptides identified highlighted. b) amino acid sequence from Spot 3 with peptides identified highlighted. Protein was digested using trypsin before MS analysis.

3.4.4 Spot 4: Uridylate Kinase

Spot 4 had peptides matching a protein on the *A. vinelandii* genome showing 25% identity (E value of 3 x 10^{-7}) to the gene product of the *pyrH* gene of *Sulfolobus solfataricus*, a uridylate kinase (GenBank # NC_002754). The peptides generated from the tryptic digest of this protein and the subsequent identification through MS are shown in Figure 3.15. According to GeneTools® this potential uridylate kinase has a predicted molecular weight of 26.6 kDa and pI of 6.1. According to the gel, the protein should have a molecular weight of approximately 25.5 kDa and a pI of 5.1. The region on the genome encoding for the uridylate kinase and the uridine monophosphate kinase, plus the upstream and downstream regions surrounding these genes are shown in Figure 3.14.

3.4.5 Spot 5: OprF

The peptides generated from Spot 5 matched a protein on the *A. vinelandii* genome showing 72% identity (E value of 1 x 10^{-142}) to the outer membrane protein OprF in *Pseudomonas resinovorans* (GenBank # AF117968). This protein has a predicted molecular weight of 37.6 kDa and pI of 5.4. The calculated molecular weight and pI, based on the two-dimensional gel, were 46.7 kDa and 4.9 respectively. In Figure 3.16 the protein sequence as well as the peptides sequenced through MS are shown. In Figure 3.17 the gene arrangement of *oprF* is shown including the upstream and downstream genes on the *A. vinelandii* genome.

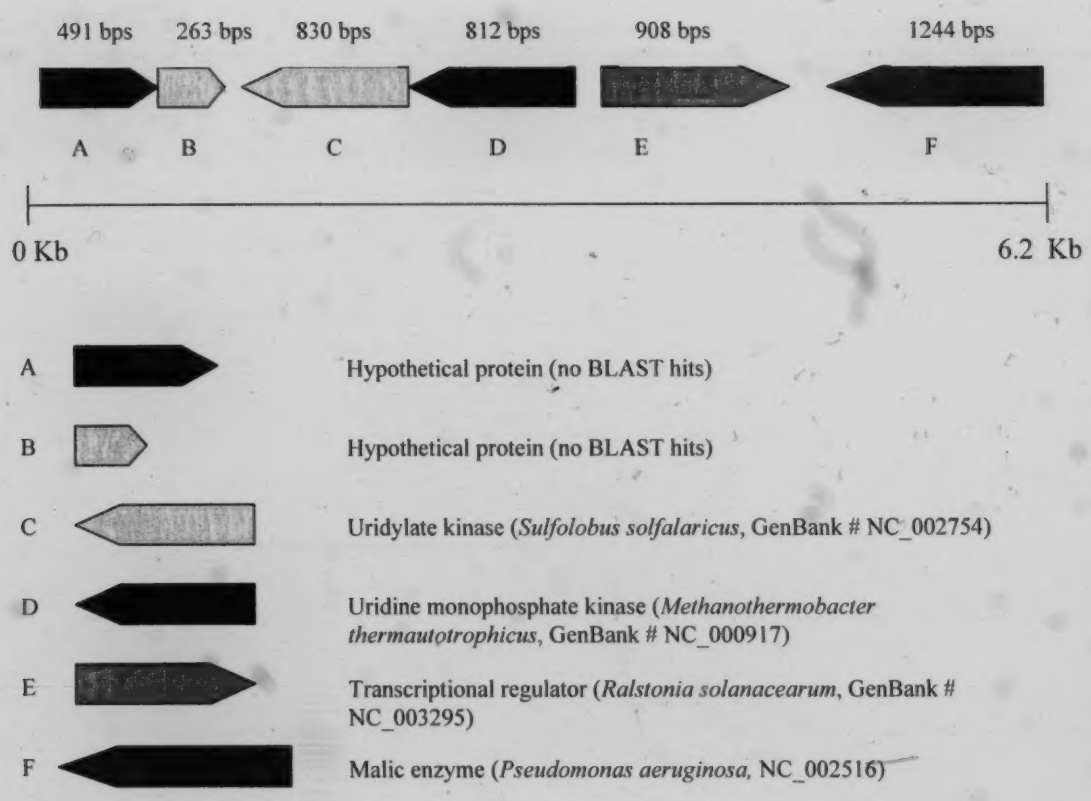


Figure 3.14. Organization of the region containing the genes encoding the uridine monophosphate protein and the uridylate kinase protein identified through two-dimensional gel electrophoresis to be regulated by RpoS in *A. vinelandii*. This fragment is found on contig 88 and spand nucleotides 69650 to 75932 on the *A. vinelandii* genome (www.azotobacter.org, 2003).

MTDTTNSIKHVISPLARQTLQDRDLTRPVAGKRPIRLLPWL
QVVKIGGRVMDRGADAILPLVEELRKLLPEHRLVIVTGAG
VRARHVFSVGLDLGLPVGSLAPLAASEAGQNGHILAAML
ASEGVSYVEHPTVADQLAIHLSATRAVVGSAFPPYHHHEF
PGSRIPPHRADTGAFLLADAFGAAGLTIVENVDGIYTADPN
GPDRGQAR[redacted]
HIER[redacted]LTAALRGEHVGTLIRTGVRPA

Figure 3.15. Amino acid sequence of the uridylate kinase protein identified from Spot 4 of the UWD two-dimensional gel. Protein was digested using trypsin before analysis. Each highlighted area represents a peptide identified through MS.

MKLKNTLGAIIGSMVAASSISALAQGQGAVEVEAFAK
HWFSDESSRDFENDEAELYGASIGYFLTDDVELVLSH
GVYRDVTTDEPIGDGGHKDIKGELTSIDALYHFGEPGA
GLRPYVSGGFAHQSIGQARGGRNNSTLANVGAGLKY
YFTDMLYARAGLEGMYNFDDDQTDWQAGVGIGLNF
GGSGGKQQPVAEPVAEVCADVDNDGVCDNVDRCPD
TPANTTVNAEGCTAVAEVVRVELDVKFDFDKAQVK
FSYADIKNLADFMKQYPQTNTTVEGHTDSVGTDAYN
QKLSERRANAVRDVLVNEYGVQGSRVDSVGYGESRP
VADNATESGRAINR

Figure 3.16. Amino acid sequence from the OprF protein identified from Spot 5 of the UWD two-dimensional gel. Protein was digested using trypsin before analysis. Each highlighted area represents a peptide identified through MS.

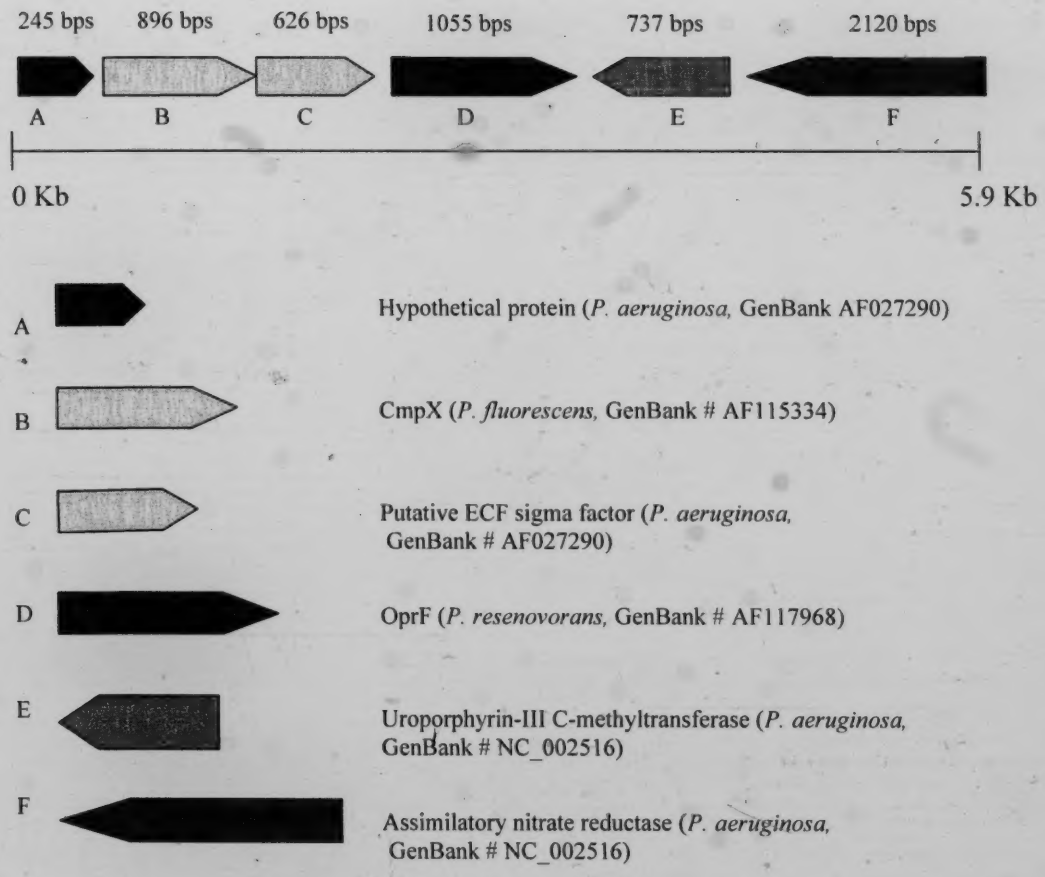


Figure 3.17. Organization of the region containing the *oprF* gene whose product was identified through two-dimensional gel electrophoresis to be regulated by RpoS in *A. vinelandii*. This fragment is found on contig 78 and spans nucleotides 45562 to 51546 on the *A. vinelandii* genome. (www.azotobacter.org, 2003)

3.4.6 Spot 6: NifU

The nitrogen fixation protein, NifU was identified from the peptides generated from Spot 6. This protein has previously been characterized in *A. vinelandii* (GeneBank # M17349) and has a predicted molecular weight (from GeneTools) of 33.3 kDa and a predicted pI of 5.9. The calculated molecular weight and pI, based on the two-dimensional were 32.2 kDa and 5.0 respectively. The protein sequence of NifU is shown in Figure 3.18 with the peptides identified through MS highlighted. The arrangement of NifU and the upstream region on the *A. vinelandii* genome is shown in Figure 3.19.

MWDYSEKVKEHFYNPKNAGAVEGANAIGDVGSLSCGDAL
RLTLKVDPETDVILDAGFQTFGCGSAIASSSALTEMVKGLT
LDEALKISNQDIADYLDGLPPEKMHCSVMGREALQAAVAN
YRGETIEDDHEEGALICKCFAVDEVMVRDTIRANKLSTVED
VTNYTKAGGGCSACHEAIERVLTEELAARGEVFVAAPIKA
KKKVK[redacted]PAAAPKLSNLQRIRRIETVLAAI
RPTLQR[redacted]NVYVKLTGACTGCQMASMTL
GGIQQR[redacted]VIPVSAAAHAQMEV

Figure 3.18. Amino acid sequence from the NifU protein identified from spot 5 of the UWD two-dimensional gel. Protein was digested using trypsin before analysis. Each highlighted peptide represents a peptide identified through MS.

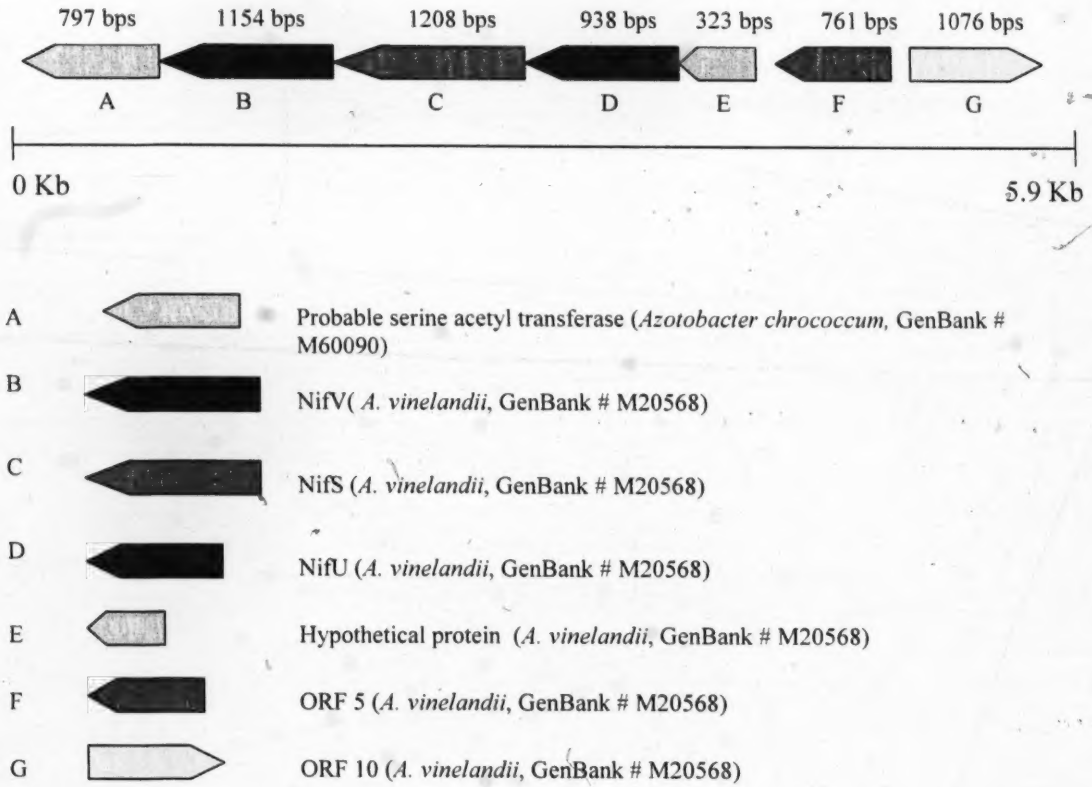


Figure 3.19. Organization of the region containing the *nifU* gene whose product was identified through 2D gel electrophoresis to be regulated by RpoS in *A. vinelandii*. This fragment is found on contig 85 and spans nucleotides 19555 to 25468 on the *A.vinelandii* genome (www.azotobacter.org, 2003)

CHAPTER 4: DISCUSSION

4.1 Growth Studies

After the RpoS mutants UWDS and 113S were constructed by Sonya Kujat Choy, growth studies were performed in order to learn more about the nature of the mutation. In *E. coli* levels of RpoS increase dramatically at the onset of stationary phase (Venturi, 2003), which also appears to be the case with *A. vinelandii* (S. Kujat-Choy, unpublished). Because of this increase, RpoS is likely playing important roles in stationary phase events. In *A. vinelandii,* stationary phase events include the increase in production of the secondary metabolites PHB, alginate and alkylresorcinol. Although *A. vinelandii* makes these compounds during exponential phase, their production and importance becomes more prevalent during stationary phase. The results of the growth studies from this work indicate that RpoS does play a role in the production of these compounds. Analysis of strain 113S showed PHB levels never reached those of the parent strain 113 over the entire course of the growth study. Similar results were found with strains UWD and UWDS, with UWDS lagging behind in PHB production and never reaching the same levels compared to the parent strain UWD. Since the intracellular storage polymer PHB is required for bacterial viability and differentiation during the stationary phase of growth (Dawes and Senior, 1973), it is not surprising that the alternative sigma factor RpoS, known to have important roles in stationary phase, plays a role in its production. An important difference between strains 113 and UWD is the alternative sigma factor, AlgU. Strain 113 has a functional *algU* gene whose product, AlgU, is responsible for the transcriptional activation of the GDP-mannose dehydrogenase gene, *algD*, a key point in the alginate biosynthetic pathway (Campos *et al.*, 1996, Page *et al.*, 2001). In strain

UWD the presence of an insertion element in the *algU* gene renders it inactive. UWD still retains the ability to form alginate, however, only during stationary phase when alginate production is independent of AlgU (Page *et al.*, 2001). What exactly controls alginate production at the level of gene expression during stationary phase is currently unknown. Previously it has been proposed (Page *et al.*, 2001) that RpoS controls alginate production during stationary phase and the results from this study further support this.

In strain 113S alginate production appears to be unaffected by the RpoS mutation as levels are very similar to the parent strain 113. In strain UWD however, this is not the case. Before day two UWD does not produce significant amounts of alginate, which is expected because of its non-functional *algU* gene. After day two however, alginate production is comparable to 113, as expected, because alginate production at this time is independent of AlgU. In strain UWDS low amounts of alginate are produced throughout the incubation period with no increase observed when alginate production becomes independent of AlgU. This supports the idea that alginate production is under the control of RpoS after entry into stationary phase. In strain 113S, after entry into stationary phase, we do not see a significant decrease in alginate. This however could be because the alginate produced during exponential phase is not being turned over and remains in the medium.

The accumulation of alkylresorcinols is typical of stationary phase *A. vinelandii* cells and is also characteristic of encysting cells (Reusch and Sadoff, 1979). The results from this study show that RpoS mutant strains UWDS and 113S are unable to produce alkylresorcinols at all. RpoS obviously plays a major role in regulating the genes responsible for the production of these compounds however, to date, how these

compounds are produced in *A. vinelandii* is unknown. It has been proposed that in *P. fluorescens* these compounds are produced via a polyketide-synthase pathway (Nowak-Thompson *et al.*, 1997), which could also be a possibility in *A. vinelandii*.

Each of the compounds PHB, alginate and alkylresorcinols are known to play roles in cells undergoing encystment. Alkylresorcinols make up a major part of the unique lipids found in encysting cells, PHB accumulates in the central body of the cysts, and alginate forms the two outer layers of the cyst, with the innermost thicker layer being the intine and the outermost thinner layer being the exine. Because 113S and UWDS are hindered in their ability to produce PHB, alginate, and alkylresorcinols, it is suspected that they do not retain the ability to form cysts. Currently it is known that strain UWD is unable to form cysts because of the low amounts of alginate produced overall by this strain, thus it is likely UWDS also cannot form cysts since it produces even less alginate that its parent. Whether strain 113 can form cysts is not known. Preliminary investigation indicates that this strain cannot form cysts, however further studies need to be done. The first step in studying the role RpoS plays in encystment is to find out definitively whether or not the parent strain 113 can form cysts. If in fact 113 can form cysts, further studies could be done in order to determine whether or not 113S retains this ability. Alternatively, an RpoS mutant could be constructed in a strain that is known to actively produce cysts such as strain 12837 (Page and Sadoff, 1975). Once this is done encystment can be induced and the effect of the RpoS mutation can be observed.

4.2 Stress Studies

In *E. coli*, there are more than 70 RpoS-dependent genes known to confer resistance against oxidative stress, near-UV irradiation, potentially lethal heat shocks, hyperosmolarity, acidic pH, ethanol and probably other stresses yet to be identified (Hengge-Aronis, 2002; Hengge-Aronis *et al.*, 1993; Jenkins *et al.*, 1990). Studies done by Jorgensen *et a.l*, (1999), have also shown that RpoS in *P. aeruginosa* confers resistance to cells exposed to hydrogen peroxide, high temperature, hyperosmolarity, low pH and ethanol, with the kill rate of stationary phase cells increasing two to three-fold in RpoS mutants compared to wild-type cells.

The results from this study indicate that RpoS is playing an important role in *A. vinelandii*'s ability to survive exposure to oxidative and hyperosmotic stress as well as during stationary phase. Like most organisms, when *A. vinelandii* encounters stress, a specific gene or sets of genes are turned on in order to confer resistance against that particular stress. Different responses can be expected when considering different organisms based on the particular environment where an organism can be found. Organisms like *E. coli*, which are typically found in the gut, are subjected to entirely different stresses compared to an opportunistic pathogen like *P. aeruginosa* or a soil bacterium like *A. vinelandii*. Therefore it is reasonable to conclude that different genes or sets of genes will be turned on or off in different organisms in response to external stress. In most Gram-negative bacteria, the "master regulator" of the stress response appears to be RpoS, however the RpoS regulon likely varies from organism to organism.

4.3 Proteomics

The goal of the proteomic study was to find and identify proteins that are regulated (directly or indirectly) by RpoS. This was done using two-dimensional electrophoresis. This task proved to be much more difficult than initially anticipated although after many months of trial and error, using various preparations and staining techniques, proteins were in fact identified to be regulated by RpoS. In addition to this, a method for preparing and visualizing proteins in two-dimensional gels was achieved.

4.3.1 Surface Layer Protein

In an attempt to find a landmark protein present in both the UWD and UWDS protein profiles, a spot was chosen for identification that appeared on both two-dimensional gels (Figure 3.7). This protein showed high homology to a para-crystalline surface layer protein in *Aeromonas hydrophila.* Surface layers (S-layers) are made up of protein or glycoprotein subunits forming regular two-dimensional crystalline arrays completely covering the bacterial cells (Bingle *et al.*, 1987; Schaffer and Messner, 2004). The protein subunits range in molecular weight from 13 to 255 kDa and assemble together by noncovalent bonds including hydrogen bonds, hydrophobic bonds and ionic bonds (Sleytr and Messner, 1983). S-layers have been identified for all major phylogenetic groups of bacteria, indicating their pivotal role for a bacterium in its natural habitat (Austin *et al.*, 1990; Schaffer and Messner, 2004). The S-layer performs important protective functions in bacteria by creating a barrier to outside threats (Koval

and Murry, 1986). S-layers have also been found to provide microorganisms with a selective advantage by functioning as a molecular sieve, molecule and ion trap or as a structure involved in cell adhesion and surface recognition. They also have been identified as contributing to virulence in pathogens and they possess potential for various biotechnological, biomedical and non-biomedical applications (Sleytr *et al.*, 1993). In general, each S-layer exhibits one of four possible two-dimensional lattice types: oblique (p1 or p2 symmetry), triangle (p3 symmetry), square or tetragonal (p4 symmetry) or hexagonal (p6 symmetry). *A. vinelandii* possess a tetragonal arranged S-layer external to the outer membrane (Bingle *et al.*, 1984). *A. vinelandii* is unique in that it has an S-layer that can be removed from whole cells or isolated outer membrane fragments by simple distilled water extraction (Schenk *et al.*, 1977). At the same time as the two dimensional work was done in this study, another student in the Page lab isolated the S-layer of *A. vinelandii* using this distilled water extraction method (Scott and Page, unpublished). This protein was subsequently sequenced at IBD using MS and the resulting protein was the same as the sequence identified in this study. The results from this study tell us that S-layer production in *A. vinelandii* is not dependent on the expression of RpoS and the S-layer protein can be used as a landmark for comparison of two-dimensional gels.

4.3.2 UMP-Kinase and Uridylate Kinase

Nucleotide monophosphate (NMP) kinases are ubiquitous enzymes that play a major role in energy metabolism, in the synthesis of nucleic acid precursors, and in the synthesis of various nucleotidyl-containing intermediates (Anderson, 1973; Neuhard and Nygaard, 1987). The reactions catalyzed by NMP-kinases are unique since they involve the direct transfer of a phosphate group from one nucleotide to another. NMP kinases bind both substrate and products simultaneously (bi-bi mechanism) (Schlichting and Reinstein, 1997). UMP-kinase is a type of NMP kinase that catalyzes the phosphorylation of UMP to UDP with ATP as the preferred donor. UMP-kinase from eukaryotic organisms is monomeric and uses both UMP and CMP as substrates with similar efficiency and shares sequence homology and similar folding with other NMP kinases (Labess *et al.*, 2002; Dreusicke *et al.*, 1988; Muller-Dieckmann and Schult, 1994). UMP-kinase from bacteria is specific for UMP and does not share homology with other NMP kinases. UMP-kinase in bacteria is arranged as a hexamer and is subjected to regulation by UTP as an inhibitor and GTP as an activator (Labesse *et al.*, 2002; Serina *et al.*, 1995). UMP-kinase in *E. coli* is encoded by the *pyrH* gene and is subject to complex regulatory mechanisms (Serina *et al.*, 1995). The results of a study done by Landais *et al.* (1999), show the dual localization of bacterial UMP kinase, with it being found in both the cytosol as well as close to the membranes in *E. coli*. This result strengthens the hypothesis of multiple functional roles of the enzyme in bacterial life. In addition to the roles described above, UMP-kinase in *E. coli* has also been described

(under the denomination *smbA*) as being involved in chromosome partitioning (Kholti *et al*, 1998; Landais *et al*, 1999).

In this study, a protein having homology to UMP-kinase, in *Methanothermobacter thermautotrophicus* was identified through MS to be regulated by RpoS in *A. vinelandii*. This protein appeared in several different spots in separate two-dimensional gel experiments. Initially this protein appeared in the UWD protein profile on the gel that was first silver stained and then destained (Section 3.2.1). Later experiments revealed this protein was present in two separate spots, both identified through MS, on the Coomassie Brilliant Blue stained gel (Section 3.3.2). Another spot on the Coomassie Brilliant Blue stained gel showed homology to a uridylate kinase in *Sulfolobus solfataricus*. According to the literature the terms uridylate kinase and UMP-kinase are interchangeable with both enzymes functioning in the same way. The reason the same protein showed up in different spots likely has to do with the nature of UMP-kinases and fact that these proteins generally form hexamers (Gagyi *et al.*, 2003). During preparation for the first dimension electrophoresis, hexamers may have only partially dissociated, from each other leading to small differences in the protein's pI (see Table 3.1). This in turn would lead to the observation of different protein spots on a two-dimensional gel.

The putative UMP-kinase and uridylate kinase identified are found next to each other on the recently sequenced *A. vinelandii* genome. Upstream of these genes is a transcriptional regulator pointing in the opposite direction (Figure 3.14). Upon examining the upstream region of all three of these genes, no consensus –10 or –35 sequences were found based on known RpoS-dependent promoters. Likely there is a

binding site upstream of the transcriptional regulator, which in turn regulates the expression of the putative UMP-kinase and the putative uridylate kinase downstream.

A gene encoding *pyrH* can be found on the *A. vinelandii* genome located separately from the putative UMP-kinase and uridylate kinase identified in this study. The *pyrH* gene product in *A. vinelandii* is 93% (E value of 1×10^{-126}) identical to the *pyrH* gene product in *P. aeruginosa*. Alignments of the UMP-kinase identified in this study with the UMP-kinase product of the *pyrH* gene in *A. vinelandii* show low homology with only 24.1% identity in a 199 amino acid overlap and an E value of 0.0011 indicating the enzymes are different (Figure 4.1). Similar results were found after aligning the putative uridylate kinase identified in this study with the UMP-kinase product of the *pyrH* gene from *A. vinelandii* with only 29.4% identity in a 109 amino acid overlap with an E value of 0.28 (Figure 4.2). A difference in theoretical pI was also observed when comparing the *pyrH* gene products of *A. vinelandii* and *P. aeruginosa* with the putative UMP-kinase and the uridylate kinase identified in this study. In *A. vinelandii* the product of the *pyrH* gene has a pI of 5.57 and in *P. aeruginosa* the pI of this protein is 5.48. This is more acidic than the pI of 6.6 observed for both the hypothetical UMP-kinase and the uridylate kinase identified in this study. The low homologies to known *pyrH* UMP-kinases as well as the differences in pI indicate the proteins found in this study are different from known *pyrH* gene products and likely play a different role in the cell.

The role of the putative UMP-kinase and uridylate kinase may possibly be linked to cell wall synthesis. UMP-kinases are important in the formation and export of sugars

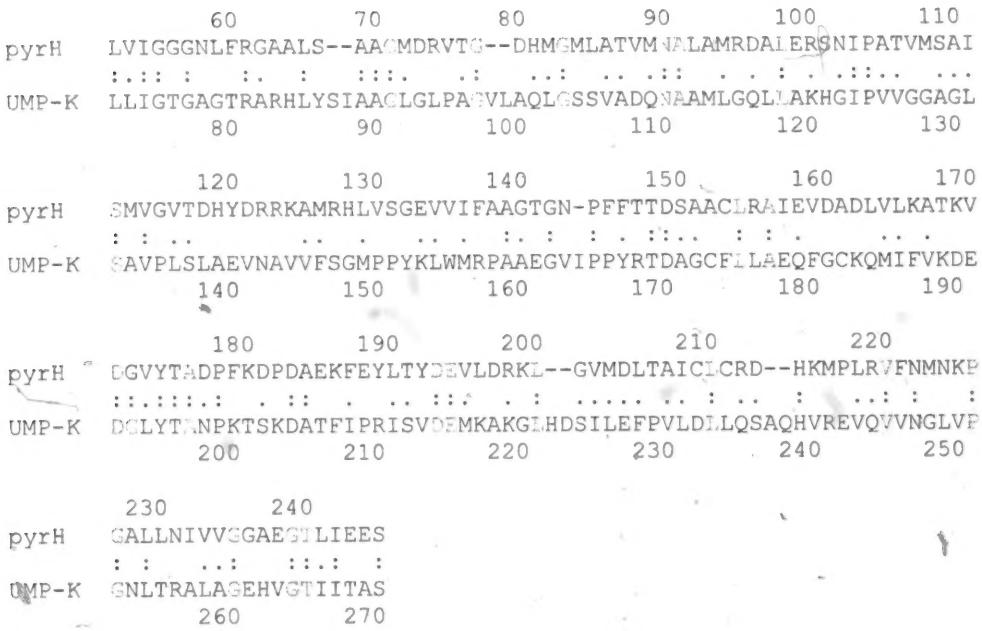


Figure 4.1 Alignment of the *pyrH* gene product from *A. vinelandii* with the putative UMP-kinase protein identified in this study. 24.1 % identity in 199 amino acid overlap, E value of 1.1 x 10^{-3}. Amino acids highlighted are conserved in the *pyrH* product of *A. vinelandii, E .coli, P. aeruginosa, Pasteurella multicida, Salmonella typhimurium, Vibrio cholerae* and *Yersinia pestis* as well as the putative UMP-kinase identified in *A. vinelandii* in this study.

```
     140       150       160       170       180       190
pyrH   GTGNPFFTTDSAACLRAIEVDADLVLKATKVDGVYTADPFKDPDAEKFEYLTYDEVLD--
       :.  :   .:..: : :    :  .  . .:::.::::: . ::  . ..:    . :
UK     GSRIPPHRADTGAFLLADAFGAAGLTIVENVDGIYTADP-NGPDRGQARFLPETSATDLA
           170       180       190       200       210       220

       200           210       220       230       240
pyrH   RKLGVMD----LTAICLCRDHKMPLRVFNMNKPGALLNIVVGGAEGTLI
       .. : .     :  .     :  ..: :  :: :   . : ::::
UK     KSEGPLPVDRALLDVMATARHIERVQVVNGLVPGRLTAALRGEHVGTLI
             230       240       250       260
```

Figure 4.2 Alignment of the *pyrH* gene product from *A. vinelandii* with the putative uridylate kinase protein identified in this study. 29.4 % identity in 109 amino acid overlap, E value of 0.28. Amino acids highlighted are conserved in the *pyrH* product of *A. vinelandii, E.coli, P. aeruginosa, Pasteurella multicida, Salmonella typhimurium, Vibrio cholerae* and *Yersinia pestis* as well as in the putative uridylate kinase identified in this study.

and any sugar containing moiety. UDP, which is formed by the phosphorylation of UMP, acts as a carrier in the formation of many polysaccharides. Polysaccharides have various functions in bacterial cells including cell wall synthesis. Peptidoglycan, which has a repetitive backbone of N-acetyl amino sugars and short peptides attached, is made in a stepwise manner by a series of enzymatic reactions using UDP. In the formation of O-specific side-chains UDP is also used and it is synthesized in a manner analogous to peptidoglycan. Core lipopolysaccharide (LPS) is synthesized using molecules of UDP-glucose or UDP-galactose (Mandelstam and McQuillen, 1973). Currently it is unknown what the synthesis of LPS and peptidoglycan is like in stationary phase *A. vinelandii* cells, but it is known that cell wall differentiation takes place, implying a function for UMP-kinase (W. J. Page, personal communication). In *E. coli*, wild-type cells tend to have short coccobacillary forms in stationary phase, whereas *rpoS*-disrupted strains have heterogeneous cell lengths and are generally larger (Lange and Hengge-Aronis, 1991b; Dougherty and Pucci, 1994). These observations suggest that RpoS protein might exert regulatory influences on components of the cell wall synthesis apparatus. Similar events occur in *A. vinelandii* with stationary phase RpoS mutant cells appearing larger and more ovoid or rod-shaped than wild-type cells (Section 3.2). Results of this study imply that the putative UMP-kinase and uridylate kinase identified may be proteins affecting cell wall synthesis and cell shape in stationary phase.

Pyrimidine synthesis also relies on the presence of UMP-kinase. Pyrimidines are synthesized from aspartic acid and carbamoyl phosphate. Cytosine nucleotides are formed from UMP via UTP. UMP must therefore be converted to UTP by UMP-kinase and UDP-kinase for this to happen. UMP is a feedback inhibitor of this pathway so

keeping levels in balance would be important (Mandelstam and McQuillen, 1973). Without UMP-kinase being expressed in RpoS mutant cells, this pathway would be thrown off-balance.

With the recent sequencing of the *A. vinelandii* genome, many hypothetical genes have been identified. The next step in identifying these genes is to find out if they are expressed. The hypothetical UMP-kinase and uridylate kinase genes do in fact give products as shown by the two-dimensional work done in this study. The putative UMP-kinase and uridylate kinase identified are likely stationary phase-specific products induced by RpoS when *A. vinelandii* is undergoing cell differentiation. If these products are specific to differentiation, it is not surprising we do not see them in other bacteria such as *E. coli* or *P. aeruginosa* as they do not undergo the same processes of differentiation leading to encystment that *A. vinelandii* does.

4.3.3 Aconitate Hydratase

Aconitases are enzymes that catalyse the interconversion of citrate and isocitrate via *cis*-aconitate in the TCA (tricarboxylic acid) and glyoxylate cycles. The TCA cycle provides a mechanism of oxidation of acetyl-CoA and is fundamental to the energetics of an organism. The glyoxylate bypass is a modification of the TCA cycle, which bypasses the two oxidative decarboxylation steps of the TCA cycle (Neidhart *et al.*, 1987). Aconitase enzymes are monomeric, containing one essential iron sulfur (FeS) center that senses low iron concentrations and oxidative stress (Tang *et al.*, 2002, Viollier *et al.*, 2001). The aconitase protein family includes the aconitases, homoaconitases,

isopropylmalate isomerases and iron regulatory proteins (Gruer *et al.*, 1997). Sequence analysis of aconitase genes from several bacteria, plants and fungi suggest the existence of two structural forms, called A and B, which differ in protein domain structure (Gruer *et al.*, 1997). The genes of both forms have been identified in several Gram-negative bacteria including *E. coli* and *Helicobacter pylori* (Schwartz *et al.*, 1999). In *E. coli* the two genetically and biochemically distinct aconitases that have been identified, AcnA and AcnB, are encoded by the *acnA* and *acnB* genes (Bradbury *et al.*, 1996). AcnB has been shown through physiological and enzymological studies to be the major TCA cycle enzyme synthesized, whereas AcnA is an enzyme specifically induced by iron or oxidative stress (Cunningham *et al.*, 1997). In response to low iron conditions or oxidative stress, AcnA binds to sequence motifs within mRNAs called iron regulatory elements and thereby provides posttranscriptional control of gene expression (Viollier *et al.*, 2001). Studies have also shown that AcnA is adapted for maintaining TCA cycle activity during exposure to and recovery from oxidative stress, whereas AcnB is adapted for its role in catabolism (Jordan *et al.*, 1999).

A protein showing homology to aconitate hydratase 2 in *P. aeruginosa* was identified in this study to be regulated by RpoS in *A. vinelandii*. Aconitate hydratase 2 is a homologue of AcnB in *E. coli*. *A. vinelandii* also has a gene encoding aconitate hydratase 1 which is a homologue of AcnA. Like *E. coli*, *A. vinelandii* may also have different roles for each enzyme, with *acnB* being expressed under normal conditions and being sensitive to oxidative stress and *acnA* being expressed during times of stress and being more resistant to oxidative stress. Proteins in this study were chosen and identified based on their presence in the wild-type organism (UWD) and their absence in the RpoS

mutant (UWDS). UWD cells were found to express the aconitate hydratase 2 gene, homologous to the *acnB* gene, whereas RpoS mutants were not. Rather than not expressing an aconitase gene at all, RpoS mutants may be expressing the more resistant aconitate hydratase 1 homologous to the *acnA* gene, given that these cells are experiencing more oxidative stress compared to the wild-type cells (See Section 3.2).

In organisms such as *E. coli* and *S. typhimurium* RpoS plays a role in the regulation of superoxide dismutase (SOD) (Nunoshiba, 1996; Fang et al., 1999), an enzyme required for dealing with toxic oxygen products. Preliminary results from *A. vinelandii* have shown that the activity of SOD in RpoS mutants is much lower than in wild-type cells (Cornish and Page, unpublished). With RpoS playing a role in SOD activity, a higher death rate for RpoS mutants exposed to oxidative stress would be expected and no expression of the more sensitive aconitase, aconitate hydratase 2, would also be expected for the mutants. These results were both observed, however, further investigation would be required to confirm this.

Toxic oxygen products are known to inactivate FeS cluster-containing enzymes including aconitase (Qurollo *et al.*, 2001). Exposure of all aconitases to reactive oxygen converts the iron-sulfur cluster from a four-iron cluster to a three-iron cluster (Jordan *et al.*, 1999; Kennedy *et al.*, 1983). Although the aconitases are very sensitive to oxygen-mediated inactivation, when oxygen is removed, enzyme activity is rapidly recovered through iron-mediated repair of the iron-sulfur cluster. Since RpoS plays a role in SOD activity in *A. vinelandii*, aconitase assembly in the RpoS mutants may be hindered given their inability to break down toxic oxygen products. This would only hold true if aconitate hydratase 1 and 2 are different from the enzymes in *E. coli* with both showing

equal sensitivity to toxic oxygen products. In this case we would not expect to see either aconitate hydratase 1 or 2 expressed in *A. vinelandii* RpoS mutant cells.

Aconitases have also been proposed to play a role in bacterial differentiation. In *Bacillus subtilis* an enzymatically inactive aconitase is able to bind mRNA and promote sporulation. As well, in *Streptomyces coelicolor* and *Streptomyces viridochromogenes*, inactivation of their aconitase genes (*acoA* and *acnA,* respectively) results in growth and developmental defects, indicating the alternative role aconitase has in cell development (Alen and Sonenshein, 1999, Voillier *et al.*, 2001). Since aconitase is a TCA cycle enzyme it is possible that it may play a role in cell differentiation leading to encystment in *A. vinelandii*. During the process of encystment when glucose is depleted, it uses stored PHB as a carbon source. Using PHB depolymerase, acetyl CoA is formed and enters the TCA or gyloxylate cycles (Page and Manchak, 1995). Aconitase is required for both of these cycles to proceed normally. The absence of the putative aconitate hydratase 2 in RpoS mutants of *A. vinelandii* may affect their ability to turn over PHB and therefore their ability to form cysts.

4.3.4 OprF

OprF is the major surface protein of the genus *Pseudomonas*. This porin plays an important role in maintenance of cell shape, in growth in a low osmolarity environment and in adhesion to various supports (Brinkman *et al.*, 1999: Gotoh *et al.*, 1989: Nicas and Hancock, 1983). OprF has been shown to form small water-filled channels (Woodruff and Hancock, 1989) and in *P. aeruginosa* it represents the major porin, permitting uptake of di- to tetrasaccharides. OprF contributes to the adherence of *P. aeruginosa* to lung epithelial cells and also facilitates its interactions with the epithelium, including colonization of the airway epithelium and the initiation of pulmonary infection (Azghani *et al.*, 2002). Because of the conserved structure of porins among *P. aeruginosa* serogroups and their antigenic capacity, outer membrane proteins, including OprF, have shown potential in the development of vaccines designed to prevent pseudomonad infections (Azghani *et al.*, 2002). In fact, a vaccine against *P. aeruginosa* based on recombinant outer membrane proteins OprF and OprI has very recently been developed (Baumann *et al.*, 2004). After intramuscular injection of this vaccine into patients with severe burns, antibodies against *P. aeruginosa* were induced. The vaccination was well tolerated and may be suitable for protection against *P. aeruginosa* infections especially in burn patients and patients with cystic fibrosis.

OprF in the Pseudomonads also has been shown to be homologous to that of the *E. coli* OmpA protein in an *E. coli lpp* (gene encoding the OmpA protein) mutant background. OprF produced from the cloned gene can substitute for OmpA in determining cell shape in such a background (Woodruff and Hancock 1989).

Immunologically, OprF and OmpA are cross-reactive and it also has been shown that both *Neisseria* spp. (Van Alphen *et al.*, 1983) and *Haemophilus influenzae* contain outer membrane proteins related to OmpA and OprF.

A protein showing homology to OprF in *P. aeruginosa* was identified in this study to be under the control of RpoS. This protein is located on an area of the *A. vinelandii* genome that is highly homologous to *P. aeruginosa*. This area in both organisms has the exact same gene arrangement (See Figure 3.16) with each gene being highly homologous to the corresponding gene in the other organism. Recently, *sigX*, a new putative ECF sigma factor has been identified in *P. aeruginosa*. This gene, located upstream of *oprF,* has been shown to play a role in OprF expression (Brinkman *et al.*, 1999). The gene immediately upstream of *oprF* in *A. vinelandii* is homologous to *sigX* of *P. aeruginosa* and also may play a role in OprF transcription in this organism.

Promoters of the *oprF* gene in *P. aeruginosa* have also been shown to be RpoD- and AlgU-dependent (Duchene *et al.*, 1988; Firoved *et al.*, 2002). AlgU (discussed in Section 4.1 and 4.2) is an alternative sigma factor required in *A. vinelandii* for encystment (Moreno *et al.,* 1998). There is an AlgU consensus sequence just upstream of the –10 region of the *oprF* gene in *P. aeruginosa* and strains lacking AlgU show much lower levels of OprF. Dual promoter sites, one dependent on AlgU and one dependent on RpoS and RpoD have been identified in *A. vinelandii*. A key enzyme in alginate biosynthesis, *algD*, has such promoter sites (Page *et al.*, 2001). The RpoS/RpoD site for this gene is weakly dependent on RpoD and strongly dependent on RpoS. OprF in *A. vinelandii* may have a dual site such as this, explaining the role of RpoS in this protein's expression.

4.3.5 NifU

The genes responsible for biological nitrogen fixation in diazotrophs are found in the *nif* (*ni*trogen *f*ixation) cluster (Arnold *et al.*, 1988). In this cluster are genes encoding enzymes responsible for the reduction of diatomic nitrogen as well as for other activities like the synthesis of cofactors and ammonia storage (Hwang *et al.*, 1996). This cluster also contains genes encoding proteins involved in the synthesis of iron-sulfur (FeS) metalloclusters, *nifU* and *nifS* (Dean *et al.*, 1993: Yuvaniyama *et al.*, 2000). FeS clusters are found in numerous proteins that have important redox, catalytic or regulatory properties and are intimately involved in the respective functions of these proteins and therefore required for stress survival of an organism. FeS clusters are known to act as electron carriers or environmental sensors or to be involved in substrate binding and activation (Yuvaniyama *et al.*, 2000). Biological FeS clusters are not formed spontaneously. A consortium of highly conserved proteins is required for both the formation of FeS clusters and their insertion into various proteins (Frazzon *et al.*, 2002). The NifU and NifS nitrogen fixation-specific gene products are involved in the acquisition of iron and sulfur necessary for the maturation of nitrogenase proteins which contain FeS clusters (Dean *et al.*, 1993).

NifS is a pyridoxal phosphate-dependent enzyme that uses L-cystine as a substrate to form an enzyme-bound cystine persufide (Zheng *et al.*, 1994: Frazzon *et al.*, 2002). Persulfides formed on NifS represent the ultimate sulfur source for nitrogenase-specific FeS cluster formation. NifU is a modular homodimeric protein that provides a molecular scaffold for the NifS-directed formation of FeS clusters (Yuvaniyama *et al.*,

2000: Frazzon *et al.*, 2002). NifU contains two identical redox FeS clusters and several conserved cysteine residues (Hwang *et al.*, 1996). Since NifS and NifU form a transient macromolecular complex, the current model is that NifS donates a sulfur to NifU and subsequent iron acquisition assembles FeS clusters on the NifU scaffold.

A. vinelandii has both NifU and NifS to assist with FeS cluster formation. In addition to these, the gene products of *iscU* and *iscS* (*i*ron-*s*ulfur *c*luster) can also be found in *A. vinelandii* and their gene products are homologues of NifU and NifS. The *iscU* and *iscS* genes are found on a widely conserved prokaryotic operon involved with general FeS cluster biosynthsis. IscS has been shown to have cysteine desulfurase activity and IscU is a truncated version of NifU, containing only the N-terminal transient-cluster binding domain identified in NifU (Krebs *et al.*, 2001). The *iscS* and *iscU* genes are often considered to have house-keeping roles involved in the general mobilization of Fe and S for FeS cluster formation whereas *nifU* and *nifS* are thought to be more specific to the formation of the nitrogenase complex.

Biological FeS clusters are among the most ancient, ubiquitous, and functionally diverse prosthetic group in all of biology. Homologues to *iscU*, *iscS*, *nifU* and *nifS* are widely conserved in nature. Homologues to these genes have been found in yeast, mouse, *Arabidopsis* and human genomes (Agar *et al.*, 2000). Hwang *et al.* (1996) have suggested that the N-terminal domain of NifU represents one of the most highly conserved protein sequence motifs in nature, having found a human gene (h*nifU*) which exhibits striking similarity to the 5' region of several *nifU* genes from both diazotrophic and non-diazotrophic organisms.

In this work, NifU was identified to be regulated by RpoS. The NifU protein appeared on the two-dimensional protein profile of UWD, but not of UWDS. Cells in this study were harvested during late stationary phase (48 h) after all glucose and ammonium in the medium had been used. At this time cells turn over their carbon reserves (PHB) and are beginning the process of encystment. Although nitrogenase genes may be derepressed in encysting *A. vinelandii* cells, they are not fixing nitrogen (Loperfido and Sadoff, 1973). Thus the NifU protein may be assisting in the assembly of FeS clusters for other proteins. Interestingly, *H. pylori* does not have the "housekeeping" genes *iscU* and *iscS* to serve the needs of the cell in the formation of FeS clusters. Instead, this organism has only two FeS cluster biosynthetic proteins, NifU and NifS, and like other organisms it has genes that encode many proteins which require FeS clusters for their proper function (Olson *et al.*, 2000). Since *H. pylori* is not a nitrogen-fixing organism, the NifU type of FeS scaffold protein is not necessarily specific to the nitrogenase protein. The absence of any other potential FeS cluster assembly genes suggests that NifS and NifU are responsible for the assembly of all FeS centers in *H. pylori*. One of the proteins in *H. pylori* predicted to contain an FeS cluster is the citric acid cycle enzyme, aconitase (Olson *et al.*, 2000). The work done in this study has shown that aconitase is not expressed in stationary phase *A. vinelandii* RpoS mutant cells (Section 3.3.2). Since the proper assembly of aconitase may require NifU, the absence of aconitase could occur because RpoS regulates *nifU* expression, which in turn plays a role in aconitase assembly rather than RpoS regulating aconitase directly. From these results it appears *A. vinelandii* may have evolved different routes in the formation of FeS clusters utilizing IscU or NifU during exponential and stationary phase growth.

4.4 Future Work

In total, five proteins were identified in this study to be present in UWD and not the corresponding *rpoS* mutant UWDS. Looking at the two-dimensional gels, the number of spots appearing on each gel is much fewer than expected and fewer than commonly seen in *E. coli* or in advertising literature. Observation of so few proteins in the gels likely has to do with the growth phase of the *A. vinelandii* cells. At 48 h, cells are in late stationary phase, having depleted glucose, and are now turning over protein and PHB, among other cell components. In short, the cells are existing in a highly degradative environment. This environment may actually cause an enrichment for the proteins important to the process of encystment and those that are stable within this environment. If particular proteins are present in high numbers in a crude cell extract, the most abundant proteins can dominate the gel, making the detection of low copy proteins difficult. This problem cannot be overcome by simply loading more protein on the gel, because the resolution will decrease and the co-migration of proteins will increase (Corthals *et al.*, 2000).

Further study of the proteins identified in this study is needed to determine more specifically the role RpoS is playing. Primer extension of the putitive UMP-kinase gene, uridylate kinase gene as well as the upstream regulator (Section 4.3.1) is necessary to show any RpoS recognition sites. This needs to be done in both an *A. vinelandii* wild-type background as well as in an *rpoS* mutant background. Promoter activity of these genes as well as other potential RpoS-regulated genes could be studied by cloning these regions into a plasmid-containing promoterless LuxAB genes upstream of a multiple

cloning site. Lux activity could then be analyzed under different conditions. Both the putative UMP-kinase gene and the uridylate kinase gene have been cloned into the pDrive (Qiagen) plasmid and subsequently called pUMPK and pUK. An antibiotic cassette could be inserted into these genes and transformed into *A. vinelandii* to inactivate the putative UMP-kinase and uridylate kinase and further explore their roles in stationary phase survival. Primer extension of the *oprF* gene also needs to be done to find out if *A. vinelandii* has two promoter sites, one regulated by AlgU and one by RpoS, as was observed for the *algD* gene (Page *et al.*, 2001). Mutations of the *iscU* and *iscS* genes involved in FeS center formation in *A. vinelandii* would give insight as to whether or not NifU is involved in FeS sulfur center formation that is not involved in nitrogenase activity. It has been found however that these *isc* genes are essential and a homozygous *iscS* mutation cannot be maintained in *A. vinelandii* (Zheng *et al.*, 1998). Alternatively, the effects of NifU during stationary phase could be observed by mutating the *nifU* gene. *In vitro* activity of NifU and NifS could also be assessed to see if they are able to assemble FeS centers specific to aconitase activity. Further study of SOD expression in wild-type and *rpoS A. vinelandii* mutant cells would confirm if this enzyme is playing a role in cell viability late into stationary phase which in turn will indicate if this enzyme is affecting aconitase assembly.

The results from this study show several areas where RpoS is exerting control. In addition to this, researchers working with *A. vinelandii* have been provided with a tool. In this study, a successful method was developed for harvesting, preparing, running and visualizing proteins from *A. vinelandii* on a two dimensional gel.

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APPENDIX A

Mass Spectrometry Results

The following pages are an example of the MS results obtained from the analysis done in this work.

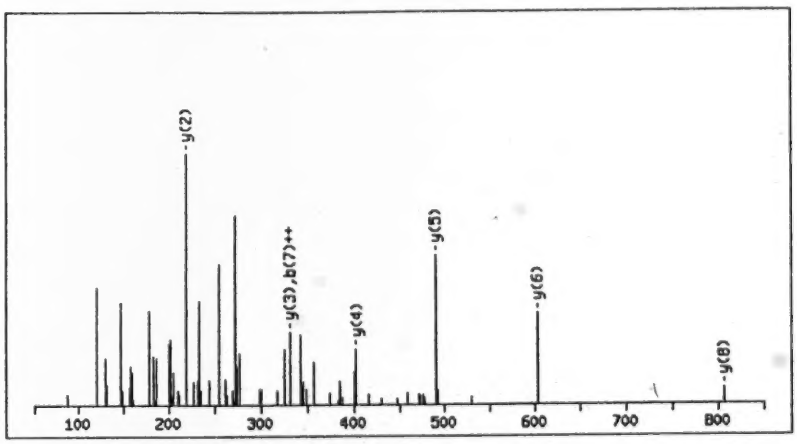
{MATRIX SCIENCE} Mascot Search Results

Peptide View

MS/MS Fragmentation of **AGFLSALAK**
Found in **gi|23062889**, (NZ_AABA01000160) hypothetical protein [Pseudomonas fluorescens]

Match to Query 2: 876.571130 from(439.287900,2+)
From data file C:\Documents and Settings\paul\Desktop\3p018_Fri\3p018_PageB1_b.pkl

Click mouse within plot area to zoom in by factor of two about that point
Or, Plot from 50 to 850 Da



Monoisotopic mass of neutral peptide Mr(calc): 876.51
Fixed modifications: Carbamidomethyl (C)
Ions Score: 56 **Expect:** 0.0055
Matches (Bold Red): 7/64 fragment ions using 12 most intense peaks

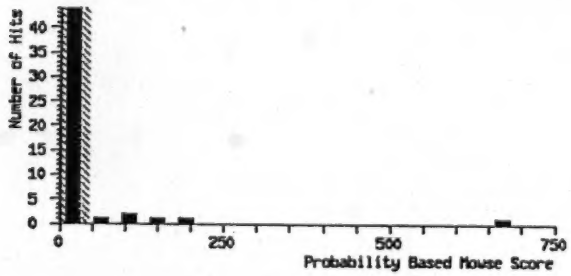
#	b	b^{++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	72.04	36.53			A							9
2	129.07	65.04			G	**806.48**	403.74	789.45	395.23	788.47	394.74	8
3	276.13	138.57			F	749.46	375.23	732.43	366.72	731.45	366.23	7
4	389.22	195.11			L	**602.39**	301.70	585.36	293.18	584.38	292.69	6
5	476.25	238.63	458.24	229.62	S	**489.30**	245.16	472.28	236.64	471.29	236.15	5
6	547.29	274.15	529.28	265.14	A	**402.27**	201.64	385.24	193.13			4
7	660.37	**330.69**	642.36	321.68	L	**331.23**	166.12	314.21	157.61			3
8	731.41	366.21	713.40	357.20	A	**218.15**	109.58	201.12	101.07			2
9					K	147.11	74.06	130.09	65.55			1

{MATRIX SCIENCE} Mascot Search Results

```
User            : Paul
Email           : paul.semchuk@ualberta.ca
Search title    : 3p018_PageB1_b.pkl
MS data file    : C:\Documents and Settings\paul\Desktop\3p018_Fri\3p018_PageB1_b.pk
Database        : NCBInr 20021030 (1226480 sequences; 390314779 residues)
Timestamp       : 14 Mar 2003 at 17:21:31 GMT
Significant hits: gi|23103554  (NZ_AAAD01000078) hypothetical protein [Azotobacter v
                  gi|23062889  (NZ_AABA01000160) hypothetical protein [Pseudomonas f
                  gi|16759155  (NC_003198) aconitate hydratase 2 (citrate hydro-lyas
                  gi|15640625  (NC_002505) aconitate hydratase 2 [Vibrio cholerae]
                  gi|23039989  (NZ_AAAU01000005) hypothetical protein [Trichodesmium
                  gi|17228762  (NC_003272) aconitate hydratase [Nostoc sp. PCC 7120]
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 45 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As [Peptide Summary] Help

Significance threshold p< 0.05 Max. number of hits 20

Standard scoring ◉ MudPIT scoring ○ Ions score cut-off 0 Show

Show pop-ups ◉ Suppress pop-ups ○ Sort unassigned [Decreasing Score] Requ

[Select All] [Select None] [Search Selected] ☐ Error tolerant [Archive Report]

1. gi|23103554 Mass: 97279 Score: 671 Queries matched: 19
(NZ_AAAD01000078) hypothetical protein [Azotobacter vinelandii]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide

☑	2	439.29	876.57	876.51	0.06	0	56	0.0055	1	AGFLSAIAK
☑	3	449.31	896.61	896.54	0.07	0	33	0.67	1	LQPGITLR
☑	4	451.80	901.59	901.52	0.07	0	37	0.61	1	LLSSVAGQK
☑	5	471.80	941.59	941.52	0.07	0	34	1.1	1	GEAASPLIG
☑	9	500.30	998.59	998.50	0.08	0	70	0.00019	1	KPEAPADSG
☑	11	530.32	1058.64	1058.56	0.08	0	65	0.00057	1	TDVLLDEVR
☑	13	622.87	1243.74	1243.64	0.10	0	46	0.044	1	VPPGVDEAA
	14	622.88	1243.75	1243.64	0.10	0	(12)	1.2e+002	3	VPPGVDEAA
☑	15	623.89	1245.77	1245.70	0.07	0	45	0.053	1	AELGLAPST
☑	16	623.90	1245.79	1245.70	0.09	0	(28)	2.8	1	AELGLAPST
☑	17	623.90	1245.80	1245.70	0.11	0	(25)	5.2	1	AELGLAPST
☑	22	649.85	1297.70	1297.60	0.10	0	54	0.0058	1	NIDTMAADI
☑	24	661.89	1321.78	1321.68	0.10	0	(36)	0.46	1	VQTGSTVVS
☑	25	661.89	1321.78	1321.68	0.10	0	84	7e-006	1	VQTGSTVVS
☑	26	688.89	1375.77	1375.67	0.11	0	23	7.8	1	YLSFDQIAE
☑	27	688.90	1375.79	1375.67	0.13	0	(20)	15	1	YLSFDQIAE
	30	753.96	1505.92	1505.81	0.12	0	13	75	10	DGIEPIQPG
☑	33	799.92	1597.83	1597.71	0.13	0	74	6.1e-005	1	MTTVGSQDT
☑	37	558.31	1671.92	1671.79	0.13	0	40	0.15	1	MLLPDTVGT

---

2. gi|23062889 **Mass: 96236 Score: 215 Queries matched: 5**
(NZ_AABA01000160) hypothetical protein [Pseudomonas fluorescens]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
2	439.29	876.57	876.51	0.06	0	56	0.0055	1	AGFLSALAK
13	622.87	1243.74	1243.64	0.10	0	46	0.044	1	VPPGVDEAA
14	622.88	1243.75	1243.64	0.10	0	(12)	1.2e+002	3	VPPGVDEAA
33	799.92	1597.83	1597.71	0.13	0	74	6.1e-005	1	MTTVGSQDT
37	558.31	1671.92	1671.79	0.13	0	40	0.15	1	MLLPDTVGT

Proteins matching the same set of peptides:
gi|23469186 **Mass: 96871 Score: 215 Queries matched: 5**
(NZ_AABH01000001) hypothetical protein [Pseudomonas syringae pv. syringae B728

---

3. gi|16759155 **Mass: 94055 Score: 165 Queries matched: 5**
(NC_003198) aconitate hydratase 2 (citrate hydro-lyase 2) [Salmonella enterica
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
11	530.32	1058.64	1058.56	0.08	0	65	0.00057	1	TDVLIDEVR
13	622.87	1243.74	1243.64	0.10	0	46	0.044	1	VPPGVDEAA
14	622.88	1243.75	1243.64	0.10	0	(12)	1.2e+002	3	VPPGVDEAA
32	791.92	1581.83	1581.71	0.12	0	15	47	2	EPILCAPND
37	558.31	1671.92	1671.79	0.13	0	40	0.15	1	MLLPDTVGT

Proteins matching the same set of peptides:
gi|16763548 **Mass:** 94040 **Score:** 165 **Queries matched:** 5
(NC_003197) aconitate hydratase 2 [Salmonella typhimurium LT2]

---

4. gi|15640625 **Mass:** 94155 **Score:** 126 **Queries matched:** 3
(NC_002505) aconitate hydratase 2 [Vibrio cholerae]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
32	791.92	1581.83	1581.71	0.12	0	15	47	2	EPILCAPND
33	799.92	1597.83	1597.71	0.13	0	74	6.1e-005	1	MTTVGSQDT
37	558.31	1671.92	1671.79	0.13	0	40	0.15	1	MLLPDTVGT

---

5. gi|23039989 **Mass:** 94856 **Score:** 124 **Queries matched:** 4
(NZ_AAAU01000005) hypothetical protein [Trichodesmium erythraeum IMS101]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
2	439.29	876.57	876.51	0.06	0	38	0.33	3	AGFLTGIAK
13	622.87	1243.74	1243.64	0.10	0	46	0.044	1	VPPGVDEAA
14	622.88	1243.75	1243.64	0.10	0	(12)	1.2e+002	3	VRPGVDEAA
37	558.31	1671.92	1671.79	0.13	0	40	0.15	1	MLLPDTVGT

---

6. gi|17228762 **Mass:** 55217 **Score:** 67 **Queries matched:** 2
(NC_003272) aconitate hydratase [Nostoc sp. PCC 7120]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
2	439.29	876.57	876.51	0.06	0	27	4.4	6	AGFLTAVAK
37	558.31	1671.92	1671.79	0.13	0	40	0.15	1	MLLPDTVGTGG

---

7. gi|15893952 **Score:** 35 **Queries matched:** 2
Sugar-binding periplasmic protein [Clostridium acetobutylicum ATCC 824]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
11	530.32	1058.64	1058.54	0.10	0	35	0.56	3	VTNNIDEVR
☑ 12	530.33	1058.66	1058.54	0.12	0	(29)	2.4	1	VTNNIDEVR

---

8. gi|21228469 **Mass:** 24164 **Score:** 33 **Queries matched:** 1
(NC_003901) hexulose-6-phosphate isomerase [Methanosarcina mazei Goe1]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑ 23	653.42	1304.83	1304.68	0.16	1	33	0.76	1	SMLQKILEGDR

9. gi|6323308 Score: 32 Queries matched: 1
Protein of unknown function, localizes to the nucleus; potential Cdc28p substr
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
11	530.32	1058.64	1058.60	0.04	1	32	1.1	4	TDVLLQDKK

10. gi|7465464 Score: 32 Queries matched: 1
cyclic beta 1-2 glucan synthetase - Brucella abortus
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
2	439.29	876.57	876.47	0.10	1	32	1.3	4	KAMASLEK

Proteins matching the same set of peptides:
gi|17988120 Score: 32 Queries matched: 1

11. gi|23135138 Score: 32 Queries matched: 1
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
9	500.30	998.59	998.58	0.01	1	32	1.2	2	KPEVKEAAK

12. gi|19703983 Mass: 58328 Score: 31 Queries matched: 1
(NC_003454) Exoenzymes regulatory protein aepA precursor [Fusobacterium nucle
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
11	530.32	1058.64	1058.63	0.01	1	31	1.5	5	TTIILDAKGK

13. gi|7293746 Score: 29 Queries matched: 1
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
2	439.29	876.57	876.44	0.13	0	29	2.6	5	AGMLAEAAK +

Proteins matching the same set of peptides:
gi|17737653 Score: 29 Queries matched: 1

14. gi|15898867 Score: 29 Queries matched: 2
Hypothetical protein SSO2081 [Sulfolobus solfataricus P2]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
11	530.32	1058.64	1058.54	0.10	1	(25)	6.4	9	KGENIDEVR
12	530.33	1058.66	1058.54	0.12	1	29	2.4	1	KGENIDEVR

---

15. gi|21741709 **Mass:** 84883 **Score:** 29 **Queries matched:** 1
(AL662946) oj991113_30.14 [Oryza sativa (japonica cultivar-group)]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
12	530.33	1058.66	1058.53	0.13	0	29	2.4	1	QWNSVGGGVR

---

16. gi|15922460 **Mass:** 35929 **Score:** 29 **Queries matched:** 1
(NC_003106) 327aa long hypothetical thioredoxin reductase [Sulfolobus tokodaii
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑ 6	478.79	955.58	955.61	-0.03	0	29	2.6	1	TILIAAGIGK

---

17. gi|22959284 **Score:** 28 **Queries matched:** 1
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
6	478.79	955.58	955.52	0.06	1	28	2.9	2	SGKAPAAGAAR

---

18. gi|11465877 **Score:** 27 **Queries matched:** 1
orf113 [Ochromonas danica]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
23	653.42	1304.83	1304.75	0.09	1	27	3.1	2	HPIKLLDDNIK

---

19. gi|6325116 **Mass:** 98203 **Score:** 27 **Queries matched:** 1
(NC_001148) Hypothetical ORF; Ypl141cp [Saccharomyces cerevisiae]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑ 19	639.37	1276.73	1276.74	-0.01	1	27	3.6	1	KPSPPSQRRPK

---

20. gi|1244658 **Score:** 26 **Queries matched:** 1
mobilization (Mob)/recombination (Pre) protein [Listeria monocytogenes]
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
11	530.32	1058.64	1058.54	0.10	1	26	5.1	6	TNPDIDKTR

Peptide matches not assigned to protein hits: (no details means no match)

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	10	517.30	1032.60	1032.52	0.07	0	20	17	1	SFAEEVVPR
☑	14	622.88	1243.75	1243.63	0.12	0	20	16	1	VPENPPSMV
☑	1	404.26	806.52	806.42	0.10	0	19	27	1	MTDIALK +
☑	7	479.76	957.53	957.42	0.10	0	19	37	1	SFNTQSMK
☑	21	647.37	1292.74	1292.68	0.06	0	18	29	1	LNFDLQVVA
☑	34	807.91	1613.81	1613.72	0.10	1	17	27	1	DTDKGEVSD
☑	35	807.92	1613.83	1613.74	0.08	1	16	36	1	EMGMKAVAV
☑	30	753.96	1505.92	1505.66	0.26	0	16	38	1	CPECEIGLT
☑	38	854.98	1707.95	1707.83	0.11	0	16	43	1	GHSESWHLS
☑	32	791.92	1581.83	1581.86	-0.03	0	15	46	1	VMPEVGAPK
☑	31	790.48	1578.96	1578.70	0.27	0	13	80	1	FQVPFTYMT
☑	18	631.39	1260.78	1260.66	0.13	0	13	1.1e+002	1	AIAALNAFN
☑	39	856.01	1710.01	1709.93	0.08	1	12	83	1	TSPVAKISP
☑	20	639.89	1277.78	1277.66	0.12	0	11	1.5e+002	1	SDLLNIGDY
☑	36	834.48	1666.95	1666.69	0.26	0	9	1.5e+002	1	EFEMSMMAS
☑	29	753.96	1505.92	1505.80	0.11	1	9	1.9e+002	1	IMDKLHGDI
☑	28	744.91	1487.81	1487.77	0.03	1	8	2.1e+002	1	IPVSPEQAR
☑	8	*481.31*	*960.61*							

Search Parameters

```
Type of search          : MS/MS Ion Search
Enzyme                  : Trypsin
Fixed modifications     : Carbamidomethyl (C)
Variable modifications  : Oxidation (M)
Mass values             : Monoisotopic
Protein Mass            : Unrestricted
Peptide Mass Tolerance  : ± 0.6 Da
Fragment Mass Tolerance: ± 0.8 Da
Max Missed Cleavages    : 1
Instrument type         : ESI-QUAD-TOF
Number of queries       : 39
```

Mascot: http://www.matrixscience.com/

APPENDIX B

B.1 *fabG* (kar) and IS101

fabG is a gene that codes for a beta-keto acyl reductase, an enzyme required in fatty acid biosynthesis in many bacteria including *P. aeruginosa* and *E. coli*. Generally, fatty acid biosynthesis genes are found on an operon with other *fab* genes, however there are exceptions, with *fabG* being found in two places on both the *P. aeruginosa* and *E. coli* genomes, once within the *fab* cluster and once on its own.

An open reading frame homologous to *fabG* was found on the *A. vinelandii* genome. This gene was not found in a cluster with other *fab* genes. In *A. vinelandii* strains UW and UWD this *fabG* homologue has an insertion element present (IS101) immediately upstream of the gene. Strain 12837, which is not related to strains UW or UWD, does not have this insertion element. This was confirmed through Southern analysis (Figure A.A.1). This gene would appear to be non-essential and may be involved in an alternate pathway like rhamnolipid synthesis. Rhamnolipids are glycolipids that reduce water surface tension and emulsify oil (Maier and Soberon-Chavez, 2000). Recently, a homologue to *fabG* was found in *P. aeruginosa* apart from the *fab* cluster (Campos-Garcia et al., 1998). This gene was found to play an important role in rhamnolipid biosynthesis and was subsequently called *rhlG*.

The next step in this work was to see if the IS element prevented *fabG* gene transcription in *A. vinelandii* UW through Northern analysis. Since it was not known under what conditions this gene may be expressed, *A. vinelandii* cells were grown in BBG with different carbon and nitrogen sources (Table A.1).

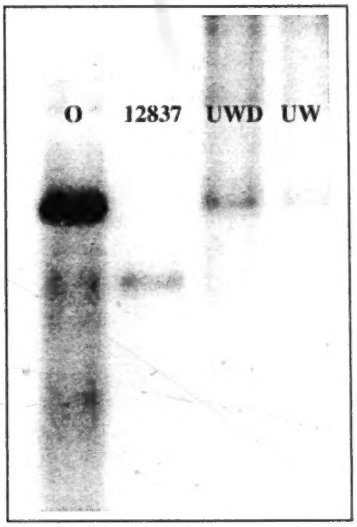


Figure A.B.1: Southern Blot probing for *fabG* homologue showing the presence of the insertion element in strains O, UWD and US. No insertion element is present in strain 12837 as indicated by the lower molecular weight shown on the gel.

Table A.B.1: Growth conditions for *A. vinelandii* strains UW and 12837. Cells were grown in these conditions for RNA harvesting. The goal was to find conditions where transcription of the *fabG* homologue was induced and whether or not IS101 was affecting transcription in strain UW.

***A. vinelandii* Strains**	**Carbon Source**	**Nitrogen Source**	**O_2 Conditions (a)**	**Time of harvesting (b)**
UW and 12827	1% glucose	Ammonium acetate	High and Low	Exp and Stat
UW and 12827	14 mM acetate	Ammonium acetate	High and Low	Exp and Stat
UW and 12827	28 mM acetate	Ammonium acetate	High and Low	Exp and Stat
UW and 12827	10 mM Valerate	Ammonium carbonate	High and Low	Exp and Stat
UW and 12827	10 mM β-hydroxybutyrate	Ammonium carbonate	High and Low	Exp and Stat

(a) Under high Oxygen conditions cells were grown in a 500 ml Erlenmeyer flask with the total volume of the media being 100 ml. Under low oxygen conditions cells were grown in 500 ml Erlenmeyer flasks with the total volume of the media being 200 ml.

(b) Cells were harvested at 12 h (Exp) and at 42 h (Stat)

Northern analysis was carried out looking for the *fabG* transcript. This was done using an internal fragment of the *fabG* homologue from *A. vinelandii* as the probe. This fragment was prepared by digesting the *fabG* homologue carried in pBluescript (plasmid name pO1) with Xho1 and Kpn1 yielding a 320 bp fragment which was subsequently random primed (Sambrook *et al.*, 1989). No transcript was found for either UW or 12837. It was thus assumed this open reading frame was either not a gene, or it was a gene expressed under unknown conditions.